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# THE JOURNAL OF GENERAL MICROBIOLOGY

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### CORRIGENDUM

BRADISH, C. J., BROOKSBY, J. B. and DILLON, J. F. (1956). *J. gen. Microbiol.*  
14, 290-314.

On page 304, Table 6, Experiment number 18  
*for 692 read 642.*





WINDER, F. G. & DENNENY, J. M. (1956). *J. gen. Microbiol.* **15**, 1-18

## Phosphorus Metabolism of Mycobacteria: Determination of Phosphorus Compounds in some Mycobacteria

By F. G. WINDER AND JOAN M. DENNENY

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**SUMMARY:** A procedure is described for the determination of phospholipid, 'phosphoprotein', 'acid-soluble' phosphate, pentosenucleic acid (DNA), deoxypentose-nucleic acid (DNA), and 'insoluble' polyphosphate in samples of mycobacteria of about 30 mg. dry weight.

The content of each of these fractions in a number of cultures of *Mycobacterium smegmatis*, *M. phlei* and *M. tuberculosis* was determined. Considerable variation in some of the fractions was found in different cultures of the one organism, especially in the case of *M. smegmatis*. The effect of the age of a culture on its content of each fraction was studied for *M. smegmatis*. Studies were made on the changes in the phosphate fractions during the first few hours after the introduction of a heavy inoculum into a fresh medium.

In the course of an investigation into the mode of action of some anti-tuberculosis phenazine pigments synthesized in these laboratories, we wished to study the effect of these pigments on the phosphorus metabolism of mycobacteria. The publications on this subject were few, and consisted of work on mycobacterial nucleic acids only (e.g. Chargaff & Saidel, 1949; Marshak & Vogel, 1951), and studies had first to be made on the adaptation of conventional techniques to the determination of the phosphorus compounds in these organisms. Since this work was started a number of papers have been published on other aspects of this subject: Ruska, Bringmann, Neckel & Schuster (1952) have shown the presence of 'metaphosphate' in *Mycobacterium avium*; Sternberg & Podoski (1953) have studied the turnover of some phosphorus-containing fractions of *M. phlei*; Meissner & Kropp (1953) and Meissner & Diller (1953) have studied the absorption of labelled phosphate by *M. tuberculosis*; Mitchell & Moyle (1954) give values for phosphorus-containing fractions of *M. phlei*. None of these papers, however, describes a critical study of methods for determining the components of the 'acid-insoluble' fraction of these bacteria.

Determinations were made of the phosphorus-containing fractions of several strains of mycobacteria. Some of the results for normally growing bacteria are given in this paper, and it is intended subsequently to publish studies on the effects of antibacterial agents on their phosphorus metabolism.

### METHODS

*Organisms used.* The strains of *Mycobacterium smegmatis* and *M. phlei* have been maintained in these laboratories for a number of years.

*Mycobacterium tuberculosis* H37Rv, H37Ra, R1Ra and Ravenel Rv were obtained from W. Steenken, Jun., Trudeau Laboratory. *M. tuberculosis*

H37RvN was a strain resistant to 100  $\mu$ g. isoniazid/ml. Its origin and characteristics are described elsewhere (Barry, Conalty, Denny, Gaffney & Winder, 1955).

*Culture and harvesting.* The basic medium used was a Proskauer & Beck medium prepared as recommended by the American Trudeau Society (1950). This medium will be referred to as P & B. The saprophytic mycobacteria were maintained on a modification of P & B which contained 6–10  $\mu$ g. ferric chloride (hydrated)/ml. In growing cultures for the experiments a small quantity of surface growth was lightly ground, suspended in P & B, and a quantity of this inoculum containing about 0.2 mg. was pipetted into 80 ml. of the modified P & B in 250 ml. conical flasks. The flasks were incubated at 37°. *Mycobacterium tuberculosis* was kept on Löwenstein-Jensen medium (containing 100  $\mu$ g. isoniazid/ml. in the case of H37RvN). Material from 2- to 3-week-old cultures was ground and inoculated, as above, into 80 ml. of P & B in 250 ml. conical flasks, which were then capped and incubated at 37°. When serum is mentioned as an addition to the medium, 5 % human serum was used.

Harvesting was carried out when the culture had reached the desired stage of growth. When material was required for the short-term growth experiments, described below, harvesting was done when there was a uniform and fairly thick pellicle over the surface of the medium, just before growth had started to slow down (e.g. 3–4 days with *Mycobacterium smegmatis*). The rate of growth, however, was subject to considerable variation and no really adequate criterion of physiological age was available. The weight of organism per unit volume of medium was variable at any stage of growth. The appearance of the pellicle on the surface gave perhaps the best idea of the stage of growth that had been reached. As a consequence of this uncertainty, in studies on the effect of age on the composition of an organism, material from the same culture had to be used throughout.

Cultures to be harvested were transferred to a Büchner funnel, and washed with distilled water. They were then suspended in distilled water by grinding for a short period in a slowly rotating Pyrex ball-mill, containing agate balls. For analysis samples containing about 30 mg. dry weight were pipetted into  $5 \times \frac{5}{8}$  in. Pyrex test tubes, in which all subsequent operations were performed. One set of samples was taken for dry weight, total nitrogen and total phosphorus determinations, and another set for fractionation.

When dealing with virulent *Mycobacterium tuberculosis* handling was carried out inside a cabinet until the stage of extraction with ethanol was reached (Fig. 1). The organisms were killed by suspending in acetone for 24 hr., followed by 24 hr. in 5 % trichloroacetic acid (TCA), so that after this period the cold TCA extracts were innocuous.

*Short-term growth experiments.* Cultures which had reached a suitable stage of growth were transferred to a Büchner funnel, washed and suspended as above. The suspension was diluted to a constant turbidity (to give about 6 mg. bacterial dry weight/ml.). The bacteria showed no appreciable loss of acid-fastness during this treatment. Forty ml. of this suspension and 2 ml. 0.5 % Tween 80 were added to 20 ml. medium contained in a 150 ml. conical flask.



The medium used had the same constituents as P & B at three times the concentration, except that equimolar sodium citrate was substituted for magnesium citrate to avoid precipitation of phosphate. The flask was plugged with cotton wool, and shaken (at about 90 cycles/min.) in a water bath at 37°. Samples (5 or 10 ml.) were withdrawn as required.

*Treatment of samples.* The samples were chilled in ice, and centrifuged in the cold. The supernatant fluid was drawn off by a fine Pasteur pipette. The organisms were washed three times by stirring up in ice-cold distilled water, using fine glass rods, the tubes were centrifuged in the cold, and the supernatant fluid drawn off. The whole procedure was carried through rapidly.

#### *Determination of dry weight, total nitrogen and total phosphorus*

Where the dry weight was to be determined the samples were pipetted into weighed tubes. After the washing, the tubes were placed in an oven at 80° overnight, allowed to cool in a desiccator and weighed. 1 ml. incinerating mixture (1:1, v/v, concentrated sulphuric acid and a strong solution of acid potassium sulphate containing 0.2% sodium selenate) and a glass bead were added. Incineration was carried out on a sand-bath according to the method of Hawes & Skavinski (1942). The tubes were then cooled, 4 ml. distilled water added and the tubes placed in a boiling water bath for about 7 min. They were then cooled, and the contents suitably diluted. Samples were taken for the determination of ammonia by microdiffusion (Conway, 1950), and for the determination of phosphate.

*Extraction of phosphorus compounds.* The bacterial mass was broken up with a glass rod. About 5 ml. acetone was added and the tube contents stirred well. The tubes were stored in the refrigerator overnight, or longer if necessary. The procedure to be described later (Fig. 1) was carried out on the samples. After each centrifuging the supernatant was pipetted off with a fine Pasteur pipette. The next extracting liquid was then added, and the same glass rods were replaced and used to suspend the bacterial residue in the liquid. The stirring was repeated several times during the longer extractions. The cold extractions were done in an ice-water mixture.

*Total phosphorus in samples and residues.* Ashing and subsequent inorganic phosphate determination were carried out as recommended by LePage (1949), but considerably longer incineration times were necessary for phospholipid and 'phosphoprotein'.

*Acid-labile phosphate.* In estimating acid-labile phosphate, 7 min. hydrolysis at 100° with N-HCl was used. It was found that the 7 min. hydrolysis was sufficient for the complete hydrolysis of the polyphosphate present in mycobacteria, though longer hydrolysis times have been adopted (Ebel, 1952). When nucleic acid was present in an extract, allowance had to be made for its hydrolysis. It was found that 23% of PNA phosphorus and 14% of DNA phosphorus were liberated by the 7 min. hydrolysis.

*Inorganic orthophosphate.* The Fiske & SubbaRow method (LePage, 1949) was employed. This method does not give completely reliable results in the

presence of acid-labile phosphate compounds. Such compounds were present along with orthophosphate in the acid-soluble fraction. But comparison of results from this method with results obtained by the method of Berenblum & Chain (1938) showed that the inaccuracy involved was small. No problem of a magnitude similar to that met by Bajaj & Krishnan (1953) was encountered.

*Ultraviolet absorption.* Measurements were carried out using a Beckman model DU spectrophotometer. All the samples contained 5% TCA and, following the recommendations of Logan, Mannell & Rossiter (1952), these were heated for 15 min. at 90° (where this had not already been done in preparing the extract). The samples were suitably diluted and read at 268.5 m $\mu$ ., where both nucleic acids have the same  $\epsilon(P)$ . The symbol  $\epsilon(P)$  is used as defined by Chargaff & Zamenhoff (1948). TCA blanks were heated and diluted in exactly the same way as the analytical solutions.

*Pentose estimation.* This was carried out according to the Mejbaum method, as modified by Patterson & Dackerman (1952). Purified mycobacterial PNA (kindly provided by Professor M. Stacey) was used to relate pentose to phosphorus.

*Deoxypentose estimation.* Deoxypentose was estimated by the Dische diphenylamine reaction as described by Patterson & Dackerman (1952), except that the colour was developed for 24 hr. at 37°. Purified mycobacterial DNA (Professor M. Stacey) was used as standard.

## EXPERIMENTAL

### *The extraction of the phosphorus compounds of Mycobacterium smegmatis*

*Acetone extraction.* Cold acetone extracted no more than 1% of the total cell phosphorus, and did not cause any further change in the various phosphorus fractions except to speed the subsequent extraction of the acid-soluble compounds. This was true even when the bacteria were stored under acetone for over a week. Acetone treatment was consequently used to stop metabolism and to permit the storage of samples.

*Extraction of acid-soluble phosphorus.* Treatment with ice-cold 5% TCA for 6 hr. was sufficient to extract the acid-soluble phosphorus compounds. Further extraction with 5% TCA in the cold did result in a very slow extraction of more phosphorus compounds, but this was due to a slow solution of nucleic acid and 'insoluble' polyphosphate. The acid-soluble compounds were not subjected to any detailed fractionation. Substances shown to be present were inorganic orthophosphate, inorganic polyphosphate (the 'soluble' metaphosphate of Juni, Kamen, Spiegelman & Wiame, 1947), and phosphorylated riboflavin derivatives. Inorganic polyphosphate constituted most of the acid-labile phosphate in these extracts. The riboflavin derivatives were also present in considerable quantity, and were responsible for most of the ultraviolet absorption of the extracts.

*Extraction of phospholipid.* Anderson and others (e.g. Lederer, 1952) have shown that the phosphatides of mycobacteria are extracted by ethanol+ether. We found that about 65% of the phosphorus that could be extracted in this



manner was removed by ethanol at room temperature, and about 30 % by a subsequent extraction with ethanol+ether at 60° for 4 min. This was usually considered adequate, but a further extraction with ethanol+ether removed practically all remaining. Meissner & Diller (1953), Sternberg & Podoski (1953) and Mitchell & Moyle (1954) used more elaborate extraction methods, but in view of the above considerations this does not seem necessary. The Reichert (1944) method, which was employed in modified form by Mitchell & Moyle (1954), extracted about the same amount of phospholipid as the above method. The Reichert method involves heating to 90°, however, and this affected the other phosphorus fractions (see the section on the Schmidt-Thannhauser method). When the Reichert method was modified by finishing the removal of methanol at room temperature *in vacuo*, it gave a considerably smaller extraction of phospholipid than before.

*Residual phosphorus.* The method of Schneider (1945) was applied to the residues. This method consists in extracting the nucleic acids with hot TCA and differential estimation of the nucleic acids by determination of ribose and deoxyribose. When this was carried out on *Mycobacterium smegmatis* the results shown in Table 1 were obtained. There was a considerable excess of pentose and phosphorus, and a smaller excess of substance estimated as deoxypentose, over the nucleic acid present as measured by ultraviolet absorption.

Table 1. *Comparison of content of phosphorus, pentose, deoxypentose, and nucleic acid in extracts prepared by treating Mycobacterium smegmatis (previously extracted with cold TCA, ethanol, and ethanol ± ether) with 5 % TCA at 90° for 15 min.*

Constituents of extract	µg. P/mg. cell N
Total P	173
Apparent DNA-P (Dische)	61
Apparent PNA-P (Mejbaum)	536
Nucleic acid P (ultraviolet absorption)	60

In order to study the excess phosphorus in purer form several attempts were made to extract it separately from the nucleic acids: (1) The cell residues were extracted with 0.01 M-bicarbonate buffer, pH 9.0, at 19° for successive periods up to a total of 24 hr. Nucleic acid and excess phosphorus were, however, extracted at a similar rate. (2) The residues were extracted with saturated aqueous urea at 37° for 24 hr., and then several times with M-NaCl at 19°. Again little separation of nucleic acid and excess phosphorus was obtained. (3) The residues were subjected to prolonged extraction with 5 % TCA at 19°. The results of such an extraction are shown in Table 2. Most of the nucleic acid was extracted during the first 24 hr., and during the remaining periods the excess phosphorus was obtained with a greatly reduced nucleic acid contamination. These extracts were useful in studying the excess phosphorus with a minimum of interference from nucleic acid.

Studies made on the above extracts showed that most of the excess phosphorus was present as inorganic polyphosphate (metaphosphate). This was

completely precipitated by barium at pH 4.5 (except for a small amount hydrolysed to orthophosphate in the case of TCA extraction), and was entirely hydrolysed to orthophosphate in 7 min. by N-HCl at 100°. It gave a meta-chromatic reaction with toluidine blue (Wiame, 1949). Paper chromatography (Crowther, 1954) suggested that a mixture of high molecular weight polyphosphates was present in the extracts. The acid-lability of this polyphosphate provided a ready means of estimating it, provided that the nucleic acid content of the solutions concerned was known so that its hydrolysis might be allowed for as described earlier.

Table 2. *Trichloroacetic acid extraction of Mycobacterium smegmatis cell residues (containing 3.20 mg. nitrogen), previously extracted with cold TCA, ethanol and ethanol + ether*

Successive extractions with	Acid-labile P ( $\mu\text{g.}$ )	Apparent nucleic acid P* ( $\mu\text{g.}$ )	Apparent RNA-P (Mejbaum) ( $\mu\text{g.}$ )	Apparent DNA-P (Dische) ( $\mu\text{g.}$ )	$\frac{E_{260}}{E_{268.5}}$
5 ml. 5% TCA at 19° for 24 hr.	194	135	142	3.1	1.07
5 ml. 5% TCA at 19° for 24 hr.	215	39.9	58.0	1.2	1.09
5 ml. 5% TCA at 19° for 24 hr.	140	10.9	40.0	2.6	1.01
5 ml. 5% TCA at 90° for 15 min.	102	38.1	588	110	0.76

\* The apparent nucleic acid P was calculated directly from the ultraviolet absorption without making allowance for extraction of DNA purines.

In order to determine pentosenucleic acid (PNA) and deoxypentose nucleic acid (DNA) use was made of the fact that the two are extracted at different rates by an acid extraction more vigorous than that used to take out the acid-soluble compounds. Ogur & Rosen (1950) used normal perchloric acid at 4° for 18 hr. This temperature of extraction was inconvenient with the facilities available to us and so 5% TCA was used as a milder agent, so that a temperature of 19° could be tried. The results of such an extraction are shown in Table 2. Obviously the Dische and Mejbaum methods are unsuitable for determining PNA and DNA in these extracts, especially in the final extract prepared at 90°, though the results with the Dische method do show that DNA is extracted to a very slight extent only by TCA at 19° for 72 hr. To obtain more conclusive results use had to be made of ultraviolet absorption studies.

On extraction of purified DNA (kindly provided by Professor M. Stacey) from *Mycobacterium phlei* with 5% TCA for 72 hr. at 19° it was found that the deoxypentose and phosphorus went into solution to a very slight extent only, but the purines were removed so that the residue was apurinic acid. When this residue was dissolved by heating with 5% TCA at 90° for 15 min., it had an absorption maximum about 275 m $\mu$ ., and the ratio of the extinction at 260 m $\mu$ . to that at 268.5 m $\mu$ . ( $E_{260}/E_{268.5}$ ) was 0.77. When purified PNA was heated in 5% TCA at 90° for 15 min. it gave an  $E_{260}/E_{268.5}$  of 1.05. When the  $E_{260}/E_{268.5}$  figures in Table 2 are examined it may be seen that the value for the residual material in *M. smegmatis* after extraction at 19° for 72 hr. corresponded



to that for apurinic acid, showing that the PNA had been removed quantitatively by the previous extractions and the DNA left as apurinic acid. Thus the DNA phosphorus in *M. smegmatis* can be estimated from the absorption at 268.5 m $\mu$ . of the heated extract from this residual material, using an  $\epsilon$  (P) of 5700 (derived from apurinic acid prepared from purified DNA as described above). The PNA phosphorus in *M. smegmatis* can be estimated from the sum of the absorptions of the 19° extracts at 268.5 m $\mu$ ., using an  $\epsilon$  (P) of 9800, quoted by Logan *et al.* (1952) and confirmed for mycobacterial PNA (kindly provided by Professor M. Stacey), but allowance must be made for the absorption of the purines from the DNA. From the data quoted, this can be done by subtracting 42 % of the calculated total DNA phosphorus.

It was found that this modified Ogur & Rosen method for determining PNA and DNA could also be applied to *Mycobacterium tuberculosis* and *M. phlei*, though some samples of these two organisms required a slightly longer extraction at 19° for the complete removal of PNA. The  $E_{260}/E_{268.5}$  ratio was always determined by extracts 'R2' and 'D' (Fig. 1) to ensure that the separation was satisfactory. If either ratio deviated slightly, the amount of contamination by the other nucleic acid could be calculated from the deviation.

There appeared to be some phosphate in these extracts which was not accounted for as nucleic acid or acid-labile phosphate (the excess 'insoluble' phosphate of Tables 3–6). This was not due to the incomplete hydrolysis of the polyphosphate in 7 min.—further hydrolysis (up to 15 min.) did not liberate it as orthophosphate, nor did its amount vary proportionately to the amount of polyphosphate present. It may be present in the extracts as glycerophosphoric acid (Mitchell & Moyle, 1951).

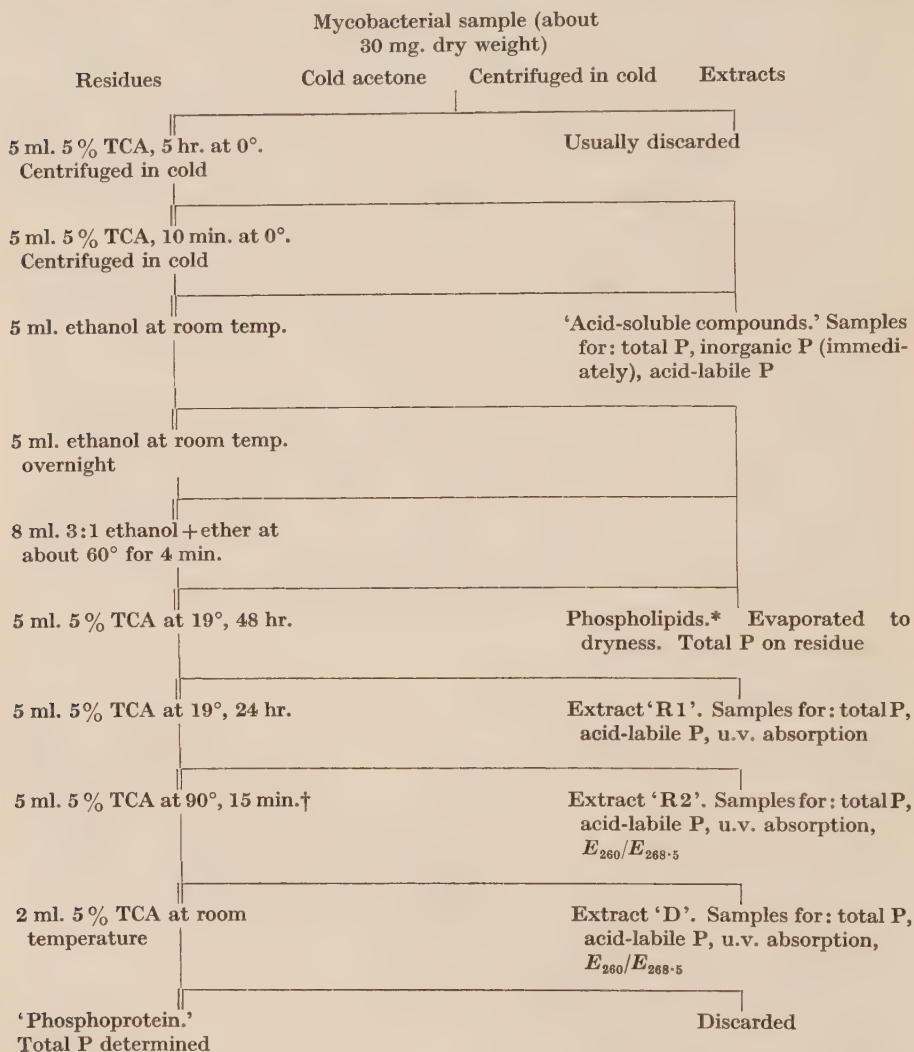
The phosphorus left in the residues after these extractions was assumed to be 'phosphoprotein' (Schneider, 1945). While in preliminary studies no nucleic acid or polyphosphate remained in the residues, it has since been found that on occasion (especially when larger samples were used) some DNA and a smaller amount of polyphosphate remained. A further extraction with 5 % TCA at 90° is therefore necessary to ensure the complete removal of these. As this was not usually done, some of the 'phosphoprotein' figures given later may be high (e.g. the higher figures in Table 6), and should therefore be taken to represent no more than an upper figure.

As a result of these studies on *Mycobacterium smegmatis* the scheme of extraction shown in the flow-sheet (Fig. 1) was adopted to obtain the results given later in this paper.

*A study of the Schmidt-Thannhauser method.* Since the Schmidt-Thannhauser method (1945) has been widely used for determining nucleic acid in bacteria, an attempt was made early in this work to apply it to mycobacteria. Difficulties due to their high lipid content and low recoveries of DNA caused it to be abandoned. From a recent re-investigation, however, it appears that it can be applied with some modification (Fig. 2).

Owing to the considerable amount of combined lipids in mycobacteria, extracted only by such drastic methods as ethanol + ether + HCl (see Lederer, 1952), it was impracticable to remove lipids completely. The subsequent

behaviour of the material in the Schmidt-Thannhauser method was the same whether the lipid extraction was carried out with ethanol-ether at 60° until no further lipid was extracted, or whether the Reichert method (1944) was used



\* For complete extraction of phospholipids a further ethanol-ether extraction at 60° should be given.

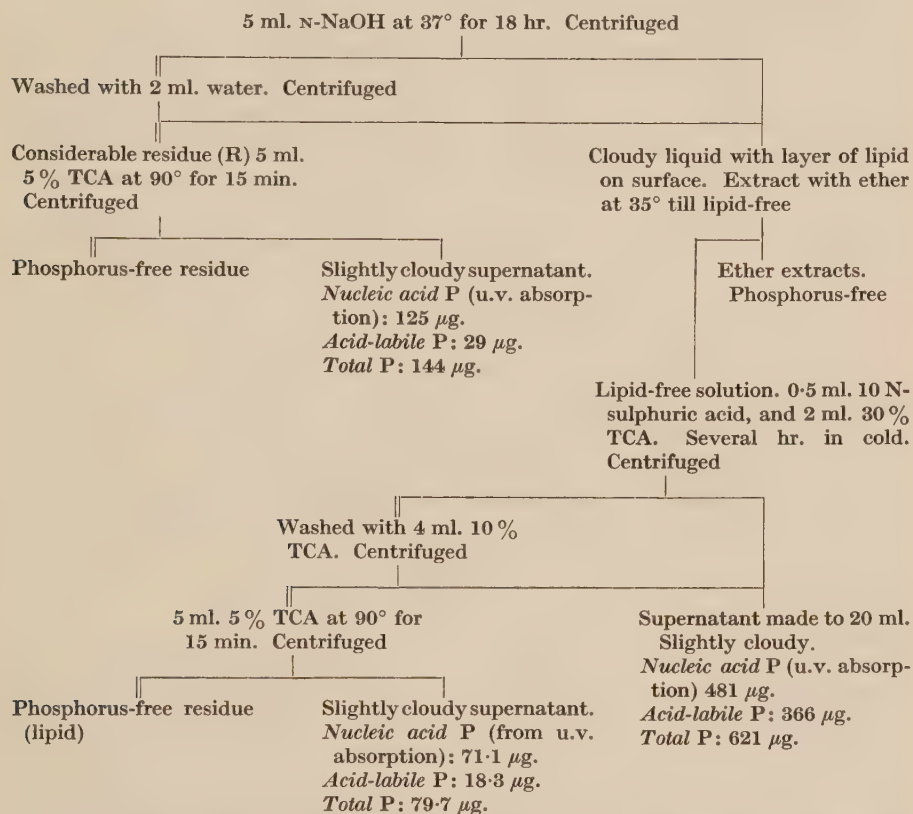
† To ensure complete removal of DNA a further extraction with TCA at 90° should be given.

Fig. 1. Flow-sheet describing extraction of phosphorus compounds from mycobacteria.

without heating to 90°, the removal of methanol being completed *in vacuo* at room temperature. The alkaline hydrolysis liberated a considerable amount of lipid, which had then to be removed by a number of extractions with warm ether. On centrifuging the suspension remaining, a substantial residue



(R, Fig. 2) and a slightly cloudy supernatant were obtained. Acidification of the supernatant gave a heavy precipitate, leaving the PNA and polyphosphate in solution. The PNA, as determined from the ultraviolet absorption of the supernatant, was in fair agreement with the figure obtained by the TCA extraction method. The discrepancy was in part due to the persistent slight



Vertical single lines indicate supernatants; vertical double lines indicate residues. Acid-labile phosphorus figures not corrected for nucleic acid hydrolysis.

Fig. 2. Application of the Schmidt-Thannhauser method to *Mycobacterium smegmatis*. *M. smegmatis* previously extracted with acetone and cold TCA; lipids then extracted after the Reichert method (1944). The material contained 400 µg. PNA-P and 227 µg. DNA-P by the method in Fig. 1.

cloudiness of this supernatant. The DNA was extracted from the precipitate with hot TCA, leaving a phosphorus-free waxy residue which, however, contained a considerable amount of ultraviolet-absorbing substances soluble in alkali—for this reason alkali was not used to dissolve the DNA. The absorption curve of the TCA solution showed that DNA only was present. It was accompanied by a little polyphosphate. The solution was again persistently slightly cloudy, so that a slight overestimation of the DNA was probable. The DNA was nevertheless much too low. Extraction of the first residue (R) with hot

TCA brought a considerable amount of nucleic acid into solution, together with a little polyphosphate. The absorption curve of this nucleic acid showed it to be DNA, and it was present in sufficient quantity to account for practically all the missing DNA. Thus the results of this method are in rough agreement with those from the acid extraction method, and suggest that with a little further investigation the Schmidt-Thannhauser method would give reliable results for mycobacteria.

When the Reichert method was employed in unmodified form (the removal of methanol being completed at 70–90°), some of the troubles due to cloudy solutions were avoided, and the residue from the alkaline extraction was nucleic acid-free. However, the heat treatment appeared to cause degradation of the DNA, so that most of it failed to precipitate on acidification but remained in solution with the PNA.

Similar results were obtained with *Mycobacterium phlei*.

*Results of determinations of phosphorus compounds  
in several strains of mycobacteria*

A number of different samples of *Mycobacterium smegmatis* were analysed by the methods which have been described, and some of the results are given in Table 3. The most striking feature of these results is their variability. The composition varies with age, as will be discussed later (Table 4), but variations observed were greater than could be explained on this basis. In fact, for all the determinations given in Table 4, material at approximately the same stage of growth was used—namely just before its multiplication rate had begun to diminish.

Observations revealed two extreme types of growth. The more usual (which we refer to as 'brittle' growth) gave a thin white film on the surface after 2 days. This thickened progressively to give a firm brittle mass which became at first faintly yellow (3 days), then a faint yellow-pink (4 days), after which it changed progressively towards light orange-brown. Rapid growth usually ended after 4 days. The medium developed only a slight yellow colour at this stage. Microscopic observation revealed rods about 4 times as long as broad, strongly acid-fast. The less usual 'leathery' growth developed in much the same way until the surface of the medium was covered. Subsequently, however, the film thickened only slightly, then took on a leathery consistency and began to sink, after which all growth stopped. The film of cells did not show any tendency to become pink, though it sometimes became yellow, and the medium became strongly yellow (the yellow colour in both cases being due to riboflavin or its derivatives, as shown by its ultraviolet absorption and fluorescence). Under the microscope the cells appeared much longer—many almost whip-like—and were less strongly acid-fast. 'Leathery' growth had characteristically much less DNA (calculated on a nitrogen basis) than 'brittle' growth.

The factors which cause the appearance of leathery growth are obscure. For long periods nothing but brittle growth was obtained, and then suddenly all the cultures would start to produce leathery growth. Sometimes a return to the parent cultures on agar produced a reversion to brittle growth, but most



frequently this failed. Originally, P & B without added ferric chloride was used for growing this organism, and it was found that the addition of about 10  $\mu\text{g./ml.}$  ferric chloride resulted in brittle growth appearing again. This addition was consequently made to the routine media. Since then, however,

Table 3. *Phosphorus-containing fractions of a number of cultures of Mycobacterium smegmatis grown on modified P & B*

Type of growth	Leathery			Brittle			
Age of culture (days)	3	4	5	4	4*	5*	5*
	$\mu\text{g. P/mg. cell N}$						
Lipid P	16.1	10.7	11.9	—	9.1	14.1	15.0
'Phosphoprotein' P	6.1	7.7	5.3	—	—	—	—
Acid-soluble P							
(i) Orthophosphate	11.4	22.7	49.8	39.9	—	22.8	13.0
(ii) Acid-labile	—	—			8.9	5.9	5.7
(iii) Stable	—	—			39.4	30.4	19.5
PNA-P	57.5	54.3	38.5	48.4	60.9	49.2	53.8
DNA-P	12.1	17.9	18.2	39.3	33.7	59.1	56.3
'Insoluble' polyphosphate	110	118	131	128	168	143	125
Excess 'insoluble' P	—	—	8.0	16.0	23.3	—	—

\* The figures for these three cultures are calculated in terms of protein nitrogen, not total nitrogen. The ratio protein nitrogen/total nitrogen was usually about 0.83.

Table 4. *Effect of age of culture on the phosphorus-containing fractions of Mycobacterium smegmatis*

Type of growth	Intermediate					Brittle		
Age of culture (days)	2	3	3.5	4	5	4	5	7
	$\mu\text{g. P/mg. cell N}$							
Lipid P	9.8	12.8	13.2	14.6	12.1	13.3	12.0	9.6
'Phosphoprotein' P	12.5	10.6	9.5	10.2	11.5	11.9	11.8	14.9
Acid-soluble P								
(i) Orthophosphate	7.8	8.8	—	8.2	9.0	8.6	8.6	19.4
(ii) Acid-labile	4.8	4.9	—	5.5	5.6	5.4	5.4	7.0
(iii) Stable	8.4	20.0	—	20.5	18.1	13.1	11.2	10.9
PNA-P	52.5	54.2	49.1	39.4	37.3	33.5	32.5	32.6
DNA-P	c. 50	29.9	23.8	23.6	22.2	51.5	52.7	54.3
'Insoluble' polyphosphate	20.2	53.0	84.0	140.3	153.0	73.4	76.0	70.0
Excess 'insoluble' phosphate	—	11.6	—	10.5	8.7	19.7	19.5	21.5

Both cultures were derived from the same inoculum and grown under identical conditions on modified P & B.

leathery growth has appeared in media modified in this way, and at present best growth is obtained when ferric chloride is omitted. On occasion, leathery growth has been obtained on fresh P & B, and brittle growth when the batch of medium used was a couple of months old, but again this was not always true. Bacterial contamination is not responsible, nor does there seem to be any question of bacteriophage being involved.

Table 4 shows the effect of age on two cultures of *Mycobacterium smegmatis* derived from the same inoculum. One of these cultures developed an intermediate type of growth, the other brittle growth. Growth in the intermediate culture began to slow down after the second day, the DNA dropped to a low level, the PNA dropped more slowly, and the polyphosphate rose rapidly. In the brittle culture a rapid rate of growth continued even up to 7 days, and it may be seen that the DNA remained at a high level, while the polyphosphate rose to a less high level than in the other culture and remained there for the duration of the experiment. Otherwise the two cultures showed a similar composition, and changed only slightly over the period studied.

Table 5. *Phosphorus-containing fractions of Mycobacterium phlei*

Medium	...	P & B	P & B	1:1 P & B- horse flesh broth
Age of culture (days)	...	3	7	3
		$\mu\text{g. P/mg. cell N}$		
Lipid P		8.3	9.8	10.5
'Phosphoprotein' P		3.9	4.3	5.8
Acid-soluble P				
(i) Total		> 100	> 100	38.8
(ii) Orthophosphate		> 100	> 100	15.5
PNA-P		26.0	20.5	35.1
DNA-P		36.2	24.5	53.5
'Insoluble' polyphosphate		6.4	18.1	17.0
Unknown 'insoluble' phosphate		11.4	8.2	10.0

*Mycobacterium phlei* was not as erratic in its growth on P & B type media as *M. smegmatis*. Some figures for the phosphorus compounds of *M. phlei* are given in Table 5. Noteworthy was the low PNA/DNA ratio—this was invariably less than 1, while in *M. smegmatis* it varied from 0.6 to nearly 5. The polyphosphate in *M. phlei* was usually very low, and never reached the levels found in *M. smegmatis*. The high level of orthophosphate found in most cultures of *M. phlei* grown on P & B is due to precipitation of magnesium ammonium phosphate from the medium. The medium as prepared is almost saturated with magnesium phosphate, and the increased alkalinity and liberation of ammonia during growth cause this precipitation. Crystals of magnesium ammonium phosphate may usually be seen attached to the underside of the pellicle. The same effect has been observed on occasion with *M. smegmatis* and *M. tuberculosis* so that in these cases, too, the orthophosphate may be overestimated. The magnesium ammonium phosphate is not removed during washing, but dissolves during the extraction of acid-soluble compounds.

Results for analyses of *Mycobacterium tuberculosis* are given in Table 6. This includes several strains of several ages, yet a fair degree of constancy of composition may be seen. Phospholipid was usually rather higher than in *M. smegmatis*. PNA was lower than in *M. smegmatis*, agreeing more with the level in *M. phlei*. The PNA/DNA ratio was 1.5 to 2.





During this research it was desired to study the changes which took place immediately after the transfer of actively growing material to a fresh culture medium. As will be seen by referring to the Methods section, the new medium had not exactly the same composition as the growth medium. Since it was made up at three times strength, magnesium was omitted to avoid phosphate precipitation on autoclaving. Consequently only traces of magnesium were present. It had a higher sodium content than the growth medium and also, as there was some magnesium phosphate precipitation in the latter medium, a slightly higher phosphate content. Further, to make the manipulation of these rather hydrophobic organisms easier, the medium was made to contain a final concentration of 0.07 % Tween 80. Previous experimentation had shown, however, that these changes did not make any difference to the behaviour of the culture during the period studied.

Table 7. *Changes relative to nitrogen in the phosphorus-containing fractions of Mycobacterium smegmatis after transfer of a 5-day old culture on modified P & B into fresh P & B*

Time after inoculation (hr.) ...	0	3	6	11.5
	mg. N/10 ml. medium			
Total cell nitrogen	2.05	2.38	2.86	3.82
	µg. P/mg. cell N			
Lipid P	14.1	13.1	11.5	10.4
'Phosphoprotein' P	5.1	5.1	5.2	5.3
Acid-soluble P				
(i) Orthophosphate + acid-labile	23.0	41.7	41.9	50.8
(ii) Stable	14.9	14.7	17.9	15.2
PNA-P	49.1	46.1	49.2	46.7
DNA-P	17.8	18.0	20.4	20.8
'Insoluble' polyphosphate	139	115	81.6	62.0

Table 7 shows the changes in such a culture of *Mycobacterium smegmatis* during the first 11 hr. The total nitrogen showed an increase within 3 hr., and had almost doubled in 11 hr. 'Phosphoprotein', PNA, and stable acid-soluble phosphate increased at about the same rate as nitrogen and DNA slightly faster. The changes in the PNA and DNA, however, varied according to their initial levels—when material with high DNA was used, DNA increased more slowly than nitrogen and PNA more rapidly. The sum of orthophosphate and acid-labile soluble phosphate increased at a greater rate than nitrogen, while phospholipid increased at a slower rate. A most striking effect was the actual decrease in insoluble polyphosphate.

When similar experiments were done using 40-day-old cultures of *Mycobacterium tuberculosis* H37Ra the changes were very much slower. Nitrogen increased by a factor of about 1.2 in 22 hr. The picture was much the same as in *M. smegmatis*, but in neither of the two experiments did polyphosphate show a decrease and in one it increased relative to nitrogen over a period of 24 hr. In one of the experiments a very striking drop in orthophosphate was



observed. This was probably due, however, to magnesium ammonium phosphate having been introduced with the inoculum (see earlier) and then slowly dissolving.

#### DISCUSSION

Table 8 gives analytical results gathered from the literature for some strains of mycobacteria. For comparison the range of our results is shown, calculated on the same basis (dry weight). This recalculation of our values is only approximate in the case of *Mycobacterium tuberculosis* and *M. phlei*. Only in the case of *M. smegmatis* was the dry weight of samples determined, and so the results for *M. phlei* and *M. tuberculosis* were recalculated assuming 12 mg. dry weight/mg. nitrogen, which is the average value for *M. smegmatis*. It will be seen that the figures from the literature are rather too fragmentary to make possible a detailed comparison with the figures in this paper. They do, however, serve to confirm that the phosphorus-containing components vary according to the strain and the cultural conditions. Some of the results, however, appear to differ from the results quoted in this paper by an amount greater than could be explained on these grounds. For example, the figures for the acid-soluble phosphate of *M. phlei* given by Sternberg & Podoski (1953) seem very high compared with the other figures in Table 8. The nucleic acid figures given by Mitchell & Moyle (1954) are very different from ours. This may be due to their having used the Schmidt-Thannhauser method without realizing its difficulties when applied to mycobacteria.

It would be interesting to know in what manner the DNA which resists extraction by the Schmidt-Thannhauser method is held. Juni, Kamen, Reiner & Spiegelman (1948) have reported that the polyphosphate of yeast is likewise bound in the alkali-insoluble residues. In the mycobacteria studied only a trace of polyphosphate is held in this fashion.

The P & B modification which was used in this work has not proved to be ideal for the study of the phosphorus compounds of the organisms. It has a very high content of magnesium so that magnesium ammonium phosphate precipitates very readily, as pH value and ammonia content of the medium rise during growth. On the other hand, this medium gives a much higher yield of cells than any other which we have tried.

Attention should be called to the fact that the values for mycobacterial PNA and DNA quoted in a previous paper (Winder & Denny, 1954) were erroneous. It was not then realized that DNA purine was extracted with PNA by 5% TCA at 19°, and that allowance must be made for this when calculating the nucleic acid values from the ultraviolet absorption figures.

During this work it was found that inorganic polyphosphate is a major phosphorus component of the strains studied (with the exception of the one culture of Ravenel Rv investigated). The substance is a high molecular weight polymer of orthophosphate, and has been referred to as 'metaphosphate' by many writers on the subject, including the present authors. However, as metaphosphoric acid properly has the formula  $(\text{HPO}_3)_n$ , the use of this term implies cyclical structure. As this has not been shown to be true of the material

Table 8. *Phosphorus-containing fractions of mycobacteria*

Organism	Culture conditions	Acid-soluble			PNA (mg. P/g. dry wt.)	DNA (mg. P/g. dry wt.)	'Insoluble', Excess poly-phosphate protein', (mg. P/g. dry wt.)			Reference	
		Lipid P (mg. P/g. dry wt.)	Ortho- phosphate (mg. P/g. dry wt.)	Other (mg. P/g. dry wt.)			(mg. P/g. dry wt.)	(mg. P/g. dry wt.)	(mg. P/g. dry wt.)		
<i>M. phlei</i>	Casein digest medium 25°, phase of retarded growth	1.15	1.52	3.29	10.11	2.78	3.91			Mitchell & Moyle (1954)	
<i>M. phlei</i>	Dubos medium, 7 days	0.45	8.7	5.7	14.9						Sternberg & Podoski (1953)
<i>M. phlei</i>	P & B-horse flesh, 3 days	0.9	1.3	1.9	2.9	4.5	1.4	0.8	0.5	This paper	
<i>M. tuberculosis</i>	Sauton's medium, 3-4 weeks	1.3-2.5	0.4-1.0			4.3-8.0				Meissner & Diller (1953)	
<i>M. tuberculosis</i>	P & B, 30-80 days	1.2-2.2	1.7-4.0	1.4-3.7	1.7-2.8	1.2-2.0	0-15.0	1.2-2.1	0.7-2.1	This paper	
<i>M. smegmatis</i>	P & B + Fe <sup>+++</sup> , 2-7 days	0.8-1.3	0.7-1.9	1.4-2.1	2.7-4.7	0.8-4.2	1.7-3.0	0.3-1.8	0.4-1.2	This paper	

in mycobacteria, and is very likely untrue, it is preferable to use the non-committal term 'polyphosphate'. Inorganic polyphosphate has been recorded as being present in a considerable number of lower plants (see review by Schmidt, 1951). It has been found in mycobacteria (Ruska *et al.* 1952; Winder & Denny, 1954) and its significance in these organisms has been discussed (Winder & Denny, 1954).

It has usually been regarded as a storage form of phosphate, and perhaps energy, being used up during periods of active growth and accumulated during resting periods. Most of the results in this paper are in keeping with this, e.g. the increase in polyphosphate with the age of a culture whose growth is slowing down (Table 4), and the drop in polyphosphate on inoculation of *Mycobacterium smegmatis* into fresh medium (Table 7). The rise in polyphosphate found on inoculating *M. tuberculosis* into fresh medium does not appear to conform with this idea. However, it may be that in this organism the anabolic reactions utilizing polyphosphates have a longer lag period than the catabolic reactions leading to its formation so that, over the immediate post-inoculation period studied, polyphosphate storage conditions would still prevail.

In subsequent papers the function of polyphosphate in mycobacteria will be discussed in the light of results obtained under abnormal growth conditions, radioactive tracers and cell-free extracts, which have given further information on the question.

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## Eight Genes Controlling the Presence or Absence of Carbohydrate Fermentation in *Saccharomyces*

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**SUMMARY:** Genes controlling carbohydrate fermentation in *Saccharomyces* often control the fermentation of more than one carbohydrate. The gene MZ has at least five different manifestations involving its ability to respond to five different carbohydrate inducers for the production of a single enzyme; a series of multiple alleles of MZ differ from one another in ability to respond to the different inducers. The gene DX controls the fermentation of dextrin and glycogen. The gene ST controls the fermentation of starch; some starch-positive cultures are Schardinger-dextrin-positive. The gene SU controls the production of a constitutive enzyme which splits both sucrose and raffinose.

### *The gene GA*

The Durham tube and melting-point tube methods (Lindegren, 1956) distinguish eight heritable differences in the fermentation of carbohydrates in the Carbondale breeding stock. The genes occur either in the dominant (capable of producing the enzyme) or the recessive (incapable of producing the enzyme) form. The gene-pair controlling galactose-fermentation (Lindegren & Lindegren, 1946) was the first analysed; the dominant form is now designated by the two-letter symbol GA (previously G), while the recessive is identified by ga (previously g). Because different genes may control the ability to ferment the same sugar a system for distinguishing phenotype and genotype has been introduced. The phenotypes are designated by three-letter abbreviations of the substrate fermented (the designation of the fermenter is by capitals and the non-fermenter by lower-case letters), while two-letter abbreviations describe the genotypes. GA controls the production of an adaptive enzyme-system which has the end result of isomerizing galactose into a utilizable hexose. Whether the enzyme system is the galactowaldenase of Cardini, Caputto, Paladini & Leloir (1950) which converts galactose-1-phosphate to glucose-1-phosphate and which needs uridine diphosphoglucose (UDPG) as coenzyme or 'galactokinase' (galactose + ATP → galactose-1-phosphate) or enzymes needed for the synthesis of UDPG has not been determined. Unadapted GA cultures (and cultures carrying ga) lack the enzyme system for this fermentation. Only one gene-pair controlling the presence or absence of ability to ferment galactose has been discovered in the Carbondale stock. Cultures in the Carbondale stock which ferment galactose on full nutrient medium within 3 days in the Durham tube and within 6 hr. in the melting-point tube are identified as GA; any culture which fails to ferment galactose under these standard conditions is identified as ga.

All the cultures of the Carbondale stock are able to ferment D-glucose and D-fructose; release of one of these hexoses from an oligosaccharide results in a positive fermentation test. Seven of the genes control the fermentation of carbohydrates by releasing fermentable hexose from an oligosaccharide. In Table 1 the genes and the substrates on which they act and some of the characteristics of the hypothetical enzymes are indicated. The vertical arrow indicates the point at which the molecule may be split.

### *The gene MZ*

MZ controls the production of the adaptive enzyme, melezitase (Palleroni & Lindegren, 1953). This gene exists in the form of a series of multiple alleles. Cells carrying the most 'complete' form of the gene are able to adapt to (and grow on) at least five different substrates: maltose, turanose, methyl- $\alpha$ -D-glucopyranoside, sucrose and melezitose. Growth on any one of these substrates produces adapted cells which are able to ferment any one of the other four and which can, in addition, split isomaltose and panose. Because of difficulty in obtaining sufficient amounts of the last two, it is not known whether there are MZ alleles conferring the capacity for growth in isomaltose and panose. One allele of MZ ( $MZ^d$ ) makes the cells capable of growth on maltose and turanose but incapable of growth on methyl- $\alpha$ -D-glucopyranoside, sucrose or melezitose, and thus a culture carrying it superficially resembles one carrying MA mz. An ma  $MZ^d$  culture can be distinguished from an Ma mz culture by transferring to melezitose (or sucrose, or methyl- $\alpha$ -D-glucopyranoside) after growth on maltose: If the culture is ma  $MZ^d$ , melezitose will be split; if it is MA mz, melezitose will not be split. The different alleles of MZ (Lindegren & Lindegren, 1953) which have been identified are indicated in the following tabulation:

Substrate	Genotype				
	MZ	MZ <sup>a</sup>	MZ <sup>b</sup>	MZ <sup>c</sup>	MZ <sup>d</sup>
Maltose	+	+	+	+	+
Turanose	+	+	+	+	+
Sucrose	+	+	+	+	—
Methyl- $\alpha$ -D-glucopyranoside	+	—	+	—	—
Melezitose	+	+	—	—	—
Raffinose	—	—	—	—	—

Melezitase splits any terminal non-reducing  $\alpha$ -D-glucopyranosyl radical from a di- or trisaccharide, irrespective of whether it is attached to another hexose or simply to a methyl group and irrespective of the type of attachments to the hexose, except that it is not active against large molecules nor against a trisaccharide such as raffinose in which the non-reducing radical is blocked.

### *The gene MA*

MA (Lindegren & Lindegren, 1949) controls the production of an adaptive enzyme system which splits maltose and turanose. A culture carrying MA mz is distinguished from one carrying MA MZ or ma MZ by the fact that the



Table 1. *Genes, enzymes and substrates*

Substrates	Enzyme	Gene
Maltose	↓ Melezitase, splits single hexoses from small molecules at the $\alpha$ -D-Gp linkage irrespective of the point of attachment of the second radical; not active against O-dextrin, nor against raffinose in which the $\alpha$ -D-Gp radical is blocked	MZ
Turanose	↓ $\alpha$ -D-Gp-(1→4) $\alpha$ -D-Gp ↓ $\alpha$ -D-Gp-(1→3) $\alpha$ -D-Fru f	
Methyl $\alpha$ -D-glucopyranoside	↓ $\alpha$ -D-Gp-(1→) Me	
Sucrose	↓ $\alpha$ -D-Gp-(1→2) $\beta$ -D-Fru f	
Melezitose	↓ $\alpha$ -D-Gp-(1→2) $\alpha$ -D-Fru f (3→1) $\alpha$ -D-Gp	
Isomaltose	↓ $\alpha$ -D-Gp-(1→6) $\alpha$ -D-Gp	
Panose	↓ $\alpha$ -D-Gp-(1→6) $\alpha$ -D-Gp (1→4) $\alpha$ -D-Gp	
Maltose	—	MA
Turanose	—	
Dextrin	—	
Glycogen	↓ $\alpha$ -D-Gp (1→4) ... (1→4) $\alpha$ -D-Gp short chain	DX
Amylopectin	↓ $\alpha$ -D-Gp (1→4) ... (1→4) $\alpha$ -D-Gp long chain	ST
Starch	↓ $\alpha$ -D-Gp-(1→2) $\beta$ -D-Fru f	
Sucrose	↓ $\alpha$ -D-Gal p-(1→6) $\alpha$ -D-Gp-(1→2) $\beta$ -D-Fru f	SU
Raffinose	↓ $\alpha$ -D-Gal p-(1→6) $\alpha$ -D-Gp	
Melibiose	↓ $\alpha$ -D-Gal p-(1→6) $\alpha$ -D-Gp	ME
D-Galactose	?	GA
Methyl $\alpha$ -D-glucopyranoside	Methyl $\alpha$ -D-glucopyranosidase	MG
Ethyl $\alpha$ -D-glucopyranoside		
Phenyl $\alpha$ -D-glucopyranoside		

MA mz cultures do not achieve adaptation to melezitose by growth on maltose. Any culture which ferments maltose and turanose, and which (after growth on maltose) does not ferment melezitose, is MA mz.

Hestrin & Lindegren (1950, 1952) found that an MA MG su mz culture when grown on maltose achieved the ability to ferment methyl- $\alpha$ -D-glucopyranoside, but that an MA mg su mz culture was incapable of heterologous adaptation. This is the only case in the fermentation genotypes of detected interaction between two genes and is interpreted to indicate that the gene MG probably functions to produce a precursor of the maltase produced by MA. This inference is based on the fact that methyl- $\alpha$ -D-glucopyranoside is very rare in nature and it seems improbable that a gene would be selected and perpetuated which produced an adaptive enzyme specific for it; it seems more probable that the gene MG functions primarily to enhance the activity of the MA gene.

#### *The gene DX*

DX (Lindegren, Lindegren, Drysdale, Hughes & Brenes-Pomales, 1956) controls the synthesis of a dextrinase capable of splitting dextrin and glycogen but neither starch nor amylopectin. The failure of dextrinase to act against the substrates split by melezitose suggests that it splits off exposed reducing radicals; its slow action against maltose suggests that it is inhibited by the proximity of a non-reducing radical.

#### *The gene ST*

The gene ST produces an amylase which splits fermentable molecules from starch and amylopectin but not from either dextrin or glycogen.

The genes DX and ST originated from a culture of *Saccharomyces diastaticus* (Andrews & Gilliland, 1952). Cultures which break down large carbohydrate polymers are exceedingly rare in *Saccharomyces*. The first *Saccharomyces* producing an enzyme of this type carried two such genes, and it is difficult to avoid the conclusion that one originated from the other. One parent of the pedigree (Tables 2 and 3) was a dextrin-fermenter originating from *S. diastaticus*. The other was descended from our inbred breeding stock. The hybrid was homozygous for the genes GA, mg and me; it was heterozygous for the genes indicated below:

Genotypes									
16119	a	DX	ST	ma	SU	AN	HI	ur	
16060	$\alpha$	dx	st	MA	su	an	hi	UR	

The fermentative phenotypes are indicated below:

Phenotypes									
16119	DXN	STA	MAL <sup>L</sup>	tur	SUC	RAF	GAL	mgs	meb
16060	dxn	sta	MAL	TUR	SUC <sup>L</sup>	raf	GAL	mgs	meb

Table 2. *Pedigree of a hybrid heterozygous for DX/dx, ST/st*

16201	dx	ST	TUR	SU	a	hi	an	
16202	dx	st	tur	su	$\alpha$	HI	AN	
16203	DX	st	TUR	su	$\alpha$	hi	AN	
16204	DX	ST	tur	SU	a	HI	an	
16206	DX	ST	TUR	su	$\alpha$	hi	an	UR
16207	Died							
16208	dx	ST	tur	su	$\alpha$	hi	an	ur
16209	dx	st	TUR	SU	a	HI	AN	UR
16210	dx	ST	tur	su	$\alpha$	hi	AN	ur
16211	dx	st	tur	su	$\alpha$	hi	AN	UR
16212	DX	st	TUR	SU	a	HI	an	UR
16213	DX	ST	TUR	SU	a	HI	an	ur
16222	DX	st	TUR	su	$\alpha$	HI	AN	ur
16223	dx	ST	tur	su	a	HI	AN	UR
16224	dx	ST?	TUR	SU	a	hi	an	UR
16225	DX	ST	tur	SU	$\alpha$	hi	an	ur
16226	dx	ST?	TUR	SU	a	hi	AN	UR
16227	DX	st	TUR	su	$\alpha$	hi	an	UR <sup>L</sup>
16228	dx	st	tur	SU	$\alpha$	hi	an	ur
16229	DX	ST?	tur	su	?	HI	AN	ur
16242	dx	ST	TUR	SU	a	HI	AN	ur
16243	DX	st	tur	su	a	hi	an	UR
16244	dx	ST	tur	SU	$\alpha$	hi	an	UR
16245	DX	ST	TUR	su	$\alpha$	HI	AN	UR
16246	DX	st	tur	SU	$\alpha$	HI	AN	ur
16247	dx	st	TUR	SU	a?	HI	AN	UR
16248	Died							
16249	dx	st	TUR	su	$\alpha$	hi	an	ur
16251	DX	st	TUR	su	a	HI	AN	ur
16252	dx	st	TUR	SU	$\alpha$	hi	an	UR
16253	dx	st	tur	su	a?	HI	AN	UR
16254	DX	st	tur	SU	$\alpha$	hi	an	.
16255	DX	st	tur	su	$\alpha$	HI	AN	ur
16256	dx	st	tur	SU	$\alpha$	hi	an	ur
16257	DX	st	TUR	su	a?	hi	an	UR
16258	dx	st	TUR	SU	a	HI	AN	UR
16259	dx	st	TUR	SU	a?	HI	an	ur
16260	DX	st	TUR	su	a	hi	AN	UR
16261	DX	ST	tur	SU	$\alpha$	HI	AN	ur
16262	dx	st	tur	su	$\alpha$	hi	an	UR
16267	dx	ST	tur	su	a?	hi	AN	ur
16268	DX	ST	TUR	SU	$\alpha$	HI	AN	UR
16269	dx	st	TUR	su	$\alpha$	hi	an	UR
16270	DX	st	tur	SU	a	HI	an	ur
16271	dx	st	.	su	$\alpha$	HI	AN	UR
16272	DX	ST	tur	SU	a	HI	AN	ur
16273	dx	ST	TUR	SU	a	hi	an	ur
16274	.	.	.	.	.	.	.	UR
16275	Died							
16276	dx	st	.	.	.	.	.	ur
16277	dx	st	.	.	.	.	.	UR
16278	DX	st	.	.	.	.	.	ur
16299	dx	ST?	TUR	su	a	hi	an	ur
16300	DX	st	tur	SU	$\alpha$	hi	an	UR
16301	dx	st	TUR	SU	a	hi	an	ur
16302	DX	st	tur	su	$\alpha$	HI	AN	ur



The abbreviations listing genotype first are DX, DXN-dextrin; ST, STA-starch; MA, MAL-maltose; TUR-turanose; SUC-sucrose; RAF-raffinose; AN-anthranilic acid; HI-histidine; UR-uracil; GA, GAL-galactose; MG, MGS-methyl- $\alpha$ -D-glucopyranoside; ME, MEB-melibiose.

16119 was turanose-negative and therefore ma. It is characteristic of cultures carrying the gene DX to produce gas slowly from maltose. This may be due to the presence of small amounts of dextrin in the maltose.

*Genetical separation of dextrinase and amylase.* Regular segregation of genes DX/dx occurred in every ascus. Although this was not true of ST/st, it is clear that DX and ST segregate independently. Since most of the cultures fermented maltose, the diagnosis for MA was made by action on turanose. Regular segregation of the other genes indicated in the pedigree show that a true hybrid was produced and that the failure of the ST gene to segregate regularly was not due to polyploidy. In Table 3 detailed analysis of the diagnosis of the genes DX and ST is given. The same fourteen asci were analysed, using the melting-point tube technique. The tubes were discarded after 7 days, and if fermentation had not occurred up to that time were registered as negatives and indicated by the minus sign.

Table 3. *Pedigree showing fermentation of carbohydrate polymers*

Each minus sign indicates a separate test. Two series of tests were made and the results are listed under A and B, respectively. The day on which fermentation occurred is indicated by Arabic numerals and if more than one positive fermentation occurred more than one numeral is listed. For instance, culture 16203 is listed as a positive fermenter of dextrin as indicated by the numerals 333. This means that three tests were made and all proved positive on the third day.

	Dextrin		Glycogen		Starch		Amylopectin		Schardinger dextrin	
	A	B	A	B	A	B	A	B	A	B
16201	≡	=	≡	=	5-	=	=	=	=	=
16202	≡	=	≡	=	=	=	4	=	=	=
16203	333	11	≡	=	=	=	=	=	=	=
16204	333	11	333	55	5-	=	=	=	5-	=
16206	333	11	555	23	5-	33	=	=	=	=
16207	Died									
16208	=	=	=	=	5-	-	44	=	=	=
16209	=	=	=	=	=	45	=	=	=	=
16210	-	=	≡	=	5-	=	5-	=	55	=
16211	≡	=	≡	=	=	=	=	=	=	=
16212	333	22	77-	=	=	=	=	=	=	=
16213	333	22	555	=	5-	=	4-	=	=	=
16222	.	.	.	.	.	.	.	.	.	.
16223	≡	=	≡	=	5-	4-	=	=	=	=
16224	=	=	=	=	=	4-	=	=	=	=
16225	333	11	333	55	5-	4	=	=	=	=
16226	≡	=	≡	=	≡	=	=	5-	=	=
16227	333	11	66-	66	=	=	=	=	=	=
16228	≡	=	=	=	=	=	=	=	=	=
16229	333	11	444	55	=	4-	=	=	=	=

Table 3 (cont.)

	Dextrin		Glycogen		Starch		Amylopectin		Schardinger dextrin	
	A	B	A	B	A	B	A	B	A	B
16242	≡	=	≡	=	77	=	=	3?	=	=
16243	333	33	666	=	=	=	=	=	=	=
16244	≡	=	≡	=	=	47	=	=	=	=
16245	222	11	33	22	5—	=	=	=	=	=
16246	22	11	66	44	=	=	=	=	=	=
16247	=	=	=	=	=	=	=	=	=	=
16248	Died									
16249	=	=	=	=	=	=	=	=	=	=
16251	333	11	333	44	=	=	=	=	=	=
16252	=	=	=	=	=	=	=	=	=	=
16253	=	=	=	=	=	=	=	=	=	=
16254	333	11	333	77	=	=	=	=	=	=
16255	.	11	.	33	.	.	.	=	.	=
16256	.	.	.	.	.	.	.	.	.	.
16257	11=	11	55=	22	.	=	.	=	=	=
16258	=	=	=	=	=	=	=	=	=	=
16259	6—	=	=	=	=	=	=	=	=	=
16260	33	11	33	55	=	=	=	=	=	=
16261	11	44	444	=	=	45	=	44	=	=
16262	=	=	.	=	.	=	.	.	.	.
16267	≡	=	≡	=	55	5	=	5	=	=
16268	333	11	333	44	55	5—	44	4—	=	=
16269	5	=	=	3	=	=	=	=	=	=
16270	333	11	≡	22	=	=	=	=	=	=
16271	≡	=	≡	=	.	=	.	=	.	=
16272	44	23	=	=	=	66	=	2	=	=
16273	=	=	=	=	=	77	55	=	=	=
16274	.	=	.	=	.	=	.	=	.	=
16275	Died									
16276	≡	=	≡	=	=	=	=	=	=	=
16277	=	=	=	=	.	=	.	=	.	=
16278	44	44	777	55	=	=	=	=	=	=
16299	=	=	=	=	=	=	=	66	=	=
16300	11	11	22	3	=	=	=	=	=	=
16301	=	=	=	=	=	=	=	=	=	=
16302	11	11	22	22	=	=	=	=	=	=

Dextrin ferments more readily than the other large polymers, occasionally showing a positive fermentation on the first day and generally showing a positive fermentation on the third day. Glycogen ferments less rapidly, no positives occurring on the first day. Whole cell preparations were used and the possibility that the same enzyme was involved in both fermentations is not excluded. A series of tests on starch and amylopectin revealed a general correspondence between the fermentability of these two substrates and indicated that dextrin-positive, glycogen-positive cultures were occasionally starch- and amylopectin-negative and, conversely, that some starch- and amylopectin-positive cultures were occasionally dextrin- and glycogen-negative. Two positive tests were obtained with Schardinger dextrin; one

of the Schardinger positives was dextrin-positive and the other dextrin-negative. These data indicate that dextrin is a reliable and precise indicator of the presence of the gene DX. The fermentation of starch and amylopectin is either so difficult or the expressiveness of the gene is so restricted that unless some special purpose can be served by analysing for this genotype, it will not be particularly helpful in mapping experiments. There are some indications that the enzymes produced by DX and ST are constitutive.

The tetatype ascus 16201-16204 produced a culture capable of fermenting both dextrin and starch (DX ST), one capable of fermenting dextrin but not starch (DX st), one incapable of fermenting dextrin but capable of fermenting starch (dx ST) and one incapable of fermenting either starch or dextrin (dx st). The pedigree was completely regular with regard to the segregation of ability to ferment dextrin; two fermenters and two non-fermenters were found in every ascus. The ability to ferment starch was irregularly segregated, some tetrads producing more than two fermenters per ascus and others not producing any fermenters. The ability to ferment turanose is the diagnostic criterion for the gene MA. It is clear that a starch fermenter in a regular tetrad such as 16201-16204 may be turanose-negative and, therefore, *ma*. Similarly, a dextrin-fermenter in a regular ascus such as 16201-16204 may be turanose-negative and, therefore, *ma*. Although these data clearly indicate the independence of the genes controlling the fermentation of starch and dextrin, they also suggest that there are diagnostic difficulties involved in analysing hybrids heterozygous for the ability to ferment starch. Amylase probably splits single hexoses from large D-glucan molecules at the 1, 4 linkage. If primary action is on 1, 4 linkages, action on 1, 6 linkages seems unlikely since the 1, 6 linkages are buried inside the polysaccharide molecule. The gene DX does not confer the ability to ferment turanose. Any dextrin-positive culture which is turanose-negative is DX *ma*, although it may act slowly on maltose.

Five *dxn STA tur* cultures (serial numbers 16208, 16210, 16223, 16244 and 16267) were studied critically for their capacity to ferment maltose and found to be maltose-negative. This observation reveals that starch-positive cultures may be completely incapable of acting on maltose, and that possession of the gene ST does not confer the ability to split maltose. It appears that starch is not disintegrated by breaking off single maltose molecules but presumably by splitting off single hexose molecules. It has been suggested that DX produces an enzyme which acts slowly on maltose because it may be inhibited by the proximity of a non-reducing radical. The complete absence of any action by the gene ST on maltose suggests that the same restriction applies to a much greater degree to the enzyme produced by ST and that it is able to split off terminal reducing radicals of starch only because they are so far removed from the non-reducing radicals. The experiment was controlled by testing the ability of the *dxn STA TUR* cultures 16299, 16273 and 16242 to act on maltose. They fermented maltose rapidly. Cultures 16249 and 16258, which are *dxn sta TUR*, were also rapid fermenters of maltose.



*The gene SU*

SU controls the production of a constitutive enzyme,  $\beta$ -D-fructofuranosidase (invertase) which splits the exposed  $\beta$ -D-fructofuranosyl radical from either sucrose or raffinose. Any culture fermenting raffinose under standard conditions is SU. Raffinose is the unique substrate which identifies SU; the enzyme produced by MZ also splits sucrose but is incapable of splitting raffinose.

*The gene ME*

ME controls the production of an adaptive enzyme splitting melibiose but not raffinose. Any melibiose-positive culture is identified as ME (Lindegren, 1949). Melibiase splits the terminal  $\alpha$ -D-glucopyranosyl radical from melibiose. It is blocked from acting on raffinose by the  $\beta$ -D-fructofuranosyl radical.

*The gene MG*

MG (Lindegren & Lindgren, 1949) controls the production of an adaptive enzyme methyl- $\alpha$ -D-glucopyranosidase; some data indicate that both phenyl- and ethyl- $\alpha$ -D-glucopyranoside are also split; the enzyme is capable of splitting non-hexose radicals from substituted  $\alpha$ -D-glucopyranosides but not from disaccharides. Any maltose-negative culture which is methyl- $\alpha$ -D-glucopyranoside-positive is identified as MG.

We are indebted to Dr Maurice Ogur for many suggestions concerning the modes of action of the gene-controlled enzymes. We have corresponded with many carbohydrate chemists and enzymologists and wish to acknowledge their helpful suggestions but hesitate to name them directly to avoid the implication that they are in agreement with the inferences made. We are indebted to Dr Allene Jeanes, Dr N. K. Richtmyer, Dr E. J. Hehre, Dr M. L. Killey, Dr R. J. Dimler, Dr W. W. Pigman and to E. R. Squibb and Sons for gifts of rare sugars.

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## Pathways of Cysteine Synthesis in *Aspergillus nidulans*

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**SUMMARY:** Cysteine was formed from sulphate, sulphite and thiosulphate as inorganic sulphur sources, by acetone-dried powders of *Aspergillus nidulans* mycelium. Added pyridoxal was obligatory for cysteine synthesis with sulphate or sulphite, and this synthesis was further enhanced by the addition of sodium pyruvate and sodium glutamate. Concomitant accumulation of cysteine sulphinic acid and utilization of glutamic acid was demonstrated. With thiosulphate, considerable synthesis of cysteine occurred in the absence of supplements, but was much enhanced by the addition of serine. Cysteine sulphinic acid did not accumulate with thiosulphate as the inorganic sulphur source. Parathiotrophic mutants, produced by ultraviolet irradiation, were used in the further elucidation of the biosynthetic mechanisms involved. Two reaction sequences: sulphate  $\rightarrow$  sulphite  $\rightarrow$  cysteine sulphinic acid  $\rightarrow$  cysteine; and thiosulphate  $\rightarrow$  cysteine-S-sulphonic acid  $\rightarrow$  cysteine probably take place in the mould.

Detailed information on sulphur metabolism in moulds was initiated by Steinberg's (1941) investigations into the utilization of various sulphur-containing compounds by *Aspergillus niger*. He concluded that the reduction of such compounds is a normal preliminary process in inorganic sulphur utilization in this mould. Hockenhull (1948) obtained similar results for *Penicillium notatum* and described two parathiotrophic mutants of this mould. Parathiotrophic mutants of *Ophiostoma multiannulatum* had previously been produced by Fries (1945) and parathiotrophy had been observed in the Saprolegniaceae by Volkonsky (1933) and in *Staphylococcus aureus* by Fildes & Richardson (1937). Hockenhull (1949) obtained a number of parathiotrophic mutants of *Aspergillus nidulans* and, from their growth behaviour on a number of compounds, postulated that sulphate was metabolized to cysteine with the intermediate formation of sulphite, sulfoxylate, thiosulphate and cysteine-S-sulphonic acid. Phinney (1948) and Phinney, Fling, Sheng & Horowitz (1950) obtained evidence that in *Neurospora crassa* sulphite, thiosulphate, cysteic acid and cysteine sulphinic acid were intermediates between sulphate and cysteine. In *Escherichia coli*, the work of Lampen, Roepke & Jones (1947) and of Cowie, Bolton & Sands (1950) indicated that sulphite, thiosulphate and possibly sulphide, were intermediates between sulphate and cysteine.

Kearney & Singer (1952, 1953) and Singer & Kearney (1954) obtained evidence that in *Proteus vulgaris* oxidation of cysteine to sulphate occurs with the intermediate formation of cysteine sulphinic acid,  $\beta$ -sulphinyl pyruvic acid and sulphite, while Chapeville & Fromageot (1954) have shown the formation of cysteine sulphinic acid from sulphite in rabbit kidney.

No experimental approach, using both metabolic studies and mutant studies simultaneously, has been made, and *Aspergillus nidulans* appears to offer



reasonable material for this purpose, as the starvation method of Pontecorvo (1953) enables large numbers of parathiotrophic mutants to be produced with relative ease. It was hoped that information gained by the above approach would make it possible to reconcile and amplify previous studies with both *A. nidulans* and other organisms.

#### METHODS

*Organisms.* *Aspergillus nidulans* (Eidam) Wint. (Commonwealth Mycological Institute Culture Collection No. 16643) was used for the metabolic experiments and as the wild-type mould for the production of mutants.

For the isolation of mutants, approximately  $20 \times 10^6$  conidia were suspended in 2 ml. 1:5000 Teepol solution, uv-irradiated in a rocked quartz dish until only 0.2% remained viable and plated out on minimal medium + cysteine. For the production of mutants by the starvation method of Pontecorvo (1953), a biotinless mutant derived from the wild type was used as the parent strain. The requirements of the parathiotrophic mutants produced were determined by the auxanographic method of Pontecorvo (1949).

*Cultivation.* The mould was grown in the following medium: 6.0 g.  $\text{NaNO}_3$ ; 0.5 g. KCl; 0.5 g.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 1.5 g.  $\text{KH}_2\text{PO}_4$ ; 20.0 g. glucose; 95  $\mu\text{g}$ .  $\text{Na}_4\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ; 393  $\mu\text{g}$ .  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ; 997  $\mu\text{g}$ .  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ; 158  $\mu\text{g}$ .  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ ; 62  $\mu\text{g}$ .  $(\text{NH}_4)_2\text{MoO}_4$ ; 8.8 mg.  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 1000 ml. water. The pH value was adjusted to 6.8 before autoclaving at 15 lb./sq.in. for 15 min. The medium was dispensed in 200 ml. amounts in 1 l. flat-bottom flasks and aerated vigorously with a fast stream of air passed through a sintered-glass distributor, while being shaken with a frequency of 150 strokes/min. at an amplitude of 4 cm.

Incubation was for 72 hr. at 20° and resulted in a dense suspension of short pieces of vegetative mycelium.

For growth studies, the mutants were grown on the above minimal medium supplemented with various sulphur compounds and biotin where necessary.

*Enzyme preparations.* The mycelium from the aerated shake culture was washed on a sintered-glass funnel with a large volume of distilled water, resuspended in 300 ml. distilled water and aerated for 5 hr. It was then filtered off and suspended in a large volume of ice-cold acetone. After standing overnight at 0°, the mycelium was again filtered on a sintered-glass funnel, washed with ice-cold acetone and ether and dried *in vacuo* over sulphuric acid. On a suggestion by Dr E. E. Snell, the mycelium was constantly aerated during all stages of manipulation until it was plunged into the ice-cold acetone. Lack of aeration during the filtration steps resulted in low and fluctuating activities in the acetone powders. Considerable endogenous production of cysteine was observed when mycelium was not starved in distilled water.

*Reaction mixture.* The total reaction mixture volume was 10 ml. consisting of 5 ml. 0.067 M-phosphate buffer (pH 7.2) in which 100–200 mg. of acetone powder were suspended. Amino and keto acids and sulphur sources were added to a final concentration of 0.1 M, as sodium salts, and pyridoxal to a final concentration of 0.01 M.

*Estimations.* At the end of each experiment, the digest was centrifuged, the precipitate washed twice with 5 ml. distilled water, and the washings and supernatant fluid combined. The precipitate was suspended in 10 ml. distilled water and heated for 10 min. in a boiling water bath. After cooling, solid material was removed by centrifugation. Cysteine and cystine were estimated directly on samples from the original supernatant fluid and on samples from the hot water extract by the method of Kassell & Brand (1938). The results were expressed as  $\mu\text{g.}$  cysteine, although the experimental manipulations led to the oxidation of varying proportions of the cysteine to cystine.

*Chromatography of amino acids.* For the production of satisfactory chromatograms it was found necessary to treat the extracts in the following manner in order to remove inorganic sulphur compounds. To a sample of the hot water extract 0.1N iodine solution was added until a faint yellow colour persisted; excess of saturated barium hydroxide solution was then added and carbon dioxide bubbled through the solution to remove excess barium hydroxide. The mixture was centrifuged and the supernatant fluid freeze-dried. After redissolving in 1–2 ml. water, 5–20  $\mu\text{l.}$  samples were chromatographed on Whatman no. 1 paper, using *n*-butanol + acetic acid + water (4:1:5) or methanol + pyridine + water (6:2:2) as solvents. The chromatograms were dipped in 0.2% (w/v) ninhydrin in acetone and the colour developed by heating at 90° for 15 min. Semi-quantitative measurements of the amount of amino acid present were made by cutting out the coloured spots, eluting with 4 ml. of acetone + water (3:1) and measuring the colour in a Beckman Model DU spectrophotometer at a wavelength of 570  $\text{m}\mu$ .

*Reagents.* Analytical grade reagents were used wherever possible. Cysteine, cystine, methionine, taurine, isethionic acid and pyridoxal were obtained from L. Light and Co.; glutamic acid, aspartic acid and serine from Roche Products Ltd.; and sodium formaldehyde sulphonylate from Brotherton and Co. Ltd. All amino acids were used in the form of their L-isomers.

Cysteine sulphinic acid was prepared according to Levine (1936), cysteic acid according to Shinohara (1932), cystine disulphoxide according to Levine (1936), cysteine-S-sulphonic acid according to Clarke (1932),  $\beta$ -sulphonyl propionic acid according to Kharasch & Brown (1940),  $\alpha$ -dihydroxy- $\beta$ -dithiolpropionic acid according to Westerman & Rose (1928) and oxalacetic acid according to Wohl & Oesterlin (1901). Sodium pyruvate was prepared from pyruvic acid (L. Light and Co.) by the method of Robertson (1942). A sample of  $\alpha$ -ketoglutaric acid was kindly provided by Mr B. Slater and a sample of  $\beta$ -sulphonyl lactic acid by Dr P. Aichenegg.

*Measurement of response of parathiotrophic mutants.* The qualitative response of the parathiotrophic mutants was tested auxanographically on the basal medium solidified with 2% agar. The quantitative response of the mutants was estimated by a spore germination method (Shepherd, unpublished) and by measurement of growth rates on agar supplemented with the compounds under test.

## RESULTS

Preliminary experiments indicated that fresh mycelium showed very low and variable activities in synthesizing cysteine from sulphate, sulphite or thio-sulphate. The activity of freeze-dried mycelium was very variable, while mycelium broken by ultrasonic disintegration, by shaking with glass beads (Mickle, 1948), by treating in the Hughes press (Hughes, 1951), or by grinding with powdered alumina (McIlwain, Roper & Hughes, 1948) showed negligible activities. Acetone powders of mycelium when prepared at a low temperature, showed a high activity which varied within the range of 38–82  $\mu\text{g}$ . cysteine produced/hr./100 mg. acetone powder with sulphite as the inorganic sulphur source, and 25–74  $\mu\text{g}$ . cysteine produced/hr./100 mg. acetone powder with thiosulphate as the inorganic sulphur source.

*Cysteine synthesis from sulphate and sulphite*

The mycelial acetone powders, when incubated alone in buffer, showed a small endogenous production of cysteine, possibly due to autolysis. As shown in Table 1, there was a considerable synthesis of cysteine when pyridoxal, glutamate and pyruvate were added to this system. The omission of glutamate, or pyruvate, caused only a 50% decrease of synthesis, as it was impossible to decrease significantly the amount of free internal amino acids in the mycelium by starvation. (After 6 hr. starvation approximately 80% of the original amount of glutamic acid still remained.) Other amino acids, such as alanine and aspartic acid, and  $\alpha$ -ketoglutaric acid and oxalacetic acid were less effective in the system. With sulphate as the inorganic sulphur source, the rate of synthesis was markedly lower, but the pattern followed was identical with that found with sulphite.

Table 1. *Cysteine synthesis by Aspergillus nidulans in the presence of sulphate and sulphite*

Acetone powder preparations of mycelium were incubated in a total volume of 10 ml. at 37° for 5 hr. Cysteine and cystine were determined in the supernatant after incubation, the results being expressed as  $\mu\text{g}$ . cysteine formed/hr./100 mg. acetone powder. Four experiments recorded.

Additions						Cysteine formed			
Pyruvate (0.1 M)	L-gluta- mate (0.1 M)	Pyridoxal (0.01 M)	SO <sub>3</sub> (0.1 M)	SO <sub>4</sub> (0.1 M)	DL-Serine (0.1 M)	Expt.			
						(1)	(2)	(3)	(4)
—	—	—	—	—	—	12	11	5	4
+	+	+	+	—	—	5	1	1	4(B)
+	+	+	—	—	—	12	5	4	7
+	+	+	+	—	—	82	38	53	58
—	+	+	+	—	—	44	34	23	34
+	—	+	+	—	—	42	32	38	34
—	—	+	+	—	—	41	30	—	—
+	+	—	+	—	—	14	12	8	10
—	—	—	+	—	+	9	7	—	—
+	+	+	—	+	—	34	21	23	30

(B signifies acetone powder suspended in buffer and heated for 10 min. at 100°.)



*Cysteine synthesis from thiosulphate*

There was a considerable synthesis of cysteine with the acetone powder, buffer and thiosulphate alone; this was markedly increased by the addition of serine. The rate of synthesis was not increased by the further addition of pyridoxal and a slight inhibition was noted upon the addition of other amino acids and keto acids (Table 2).

Table 2. *Cysteine synthesis by Aspergillus nidulans in the presence of thiosulphate*

Acetone powder preparations of mycelium were incubated in a total volume of 10 ml. at 37° for 5 hr. Cysteine and cystine were determined in the supernatant after incubation, the results being expressed as  $\mu\text{g.}$  cysteine formed/hr./100 mg. acetone powder. Two experiments recorded.

Additions					Cysteine formed	
Pyruvate (0.1 M)	L-Glutamate (0.1 M)	Pyridoxal (0.01 M)	S <sub>2</sub> O <sub>3</sub> (0.1 M)	DL-Serine (0.1 M)	Expt.	
					(1)	(2)
—	—	—	—	—	11	5
—	—	—	+	—	42	55
—	—	—	+	+	68	74
+	+	+	+	+	48	51
—	—	+	+	—	45	51
—	—	—	+	+	9	7 (B)

(B signifies acetone powder suspended in buffer and heated for 10 min. at 100°.)

*Cysteine sulphinic acid as an intermediate in the synthesis*

The production of cysteine sulphinic acid and the utilization of glutamic acid were demonstrated with sulphite as the inorganic sulphur source. With thiosulphate as the sulphur source, formation of cysteine sulphinic acid was not observed. The results obtained by a semi-quantitative estimation of changes in free internal amino acids, other than cysteine, are shown in Table 3.

Table 3. *Formation and utilization of amino acids during cysteine synthesis*

Acetone powder preparations of mycelium were incubated in a total volume of 10 ml. at 37° for 5 hr. Amino acids were determined after incubation of hot water extracts of the acetone powders by the chromatographic method described in the text, the results being expressed in arbitrary units.

Additions						Amino acids present	
Pyruvate (0.1 M)	L-Glutamate (0.1 M)	Pyridoxal (0.01 M)	SO <sub>3</sub> (0.1 M)	S <sub>2</sub> O <sub>3</sub> (0.1 M)	DL-Serine (0.1 M)	Cysteine sulphinic acid	Glutamate
—	—	—	—	—	—	44	212
+	+	+	+	—	—	35	850 (B)
+	+	+	+	—	—	112	713
—	+	+	+	—	—	57	742
+	—	+	+	—	—	71	175
—	—	—	—	+	—	30	225
+	+	+	—	+	—	30	818
—	—	—	—	+	+	41	214
—	—	—	—	+	+	41	112 (B)

(B signifies acetone powder suspended in buffer and heated for 10 min. at 100°.)

*Growth studies with mutant organisms*

The results of growth studies with various mutants, by the colony diameter method, are shown in Table 4. Auxanographic tests gave results similar to those obtained by this method. In addition to the various mutants mentioned in the text, forty-two other mutants were obtained, the metabolic block in all cases being between sulphate and sulphite. Mutants 1 and 7 made relatively poor growth on all media, whereas the growth of the other mutants, where it occurred, was more like that of the wild type.

Table 4. *Growth rates of parathiotrophic mutants on various sulphur sources*

Figures express colony diameter in mm. after incubation at 37° for 48 hr.

Sulphur compound (100 µg./ml. medium)	Mutant nos.					Wild- type
	2	8	9	1	7	
Sulphate	7	5	0	0	0	28
Sulphite	18	14	0	0	4	28
Thiosulphate	21	13	16	7	4	27
Cysteine sulphinic acid	18	15	5	7	10	31
Cysteic acid	24	14	0	10	10	29
Cysteine-S-sulphonic acid	23	10	13	7	11	29
Taurine	26	14	0	10	11	30
β-Sulphonyl lactic acid	24	13	0	8	11	28
Formaldehyde sodium sulfoxylate	23	15	0	2	10	34
β-Sulphonyl propionic acid	20	16	0	10	9	31
α-Dihydroxy-β-dithiolpropionic acid	18	16	11	8	10	32
Cystine disulphoxide	19	15	17	11	8	26
Formaldehyde bisulphite	18	15	0	0	9	29
Isethionic acid	24	15	0	0	11	18
Cysteine	29	16	21	10	12	29

*Studies with mutant no. 7*

Mutant no. 7 was studied in greatest detail because of the interest aroused by its poor growth on thiosulphate. The results of the metabolic experiments indicate that sulphite and thiosulphate may be metabolized by different paths, and it might be expected that mutants would be found which could utilize sulphite but not thiosulphate, and vice versa. That mutant no. 7 does not utilize either sulphite or thiosulphate to any large extent may indicate that these compounds are on the same pathway, or that some type of double block in two separate pathways is present. The response of this mutant to cysteine is shown in Fig. 1, and the quantitative response to various other sulphur compounds is shown in Fig. 2, as measured in spore germination tests.

*Apparent 'leakiness' of the mutants*

Table 5 illustrates 'leakiness', i.e. some growth and germination on sulphate, but much less than that of the wild type. Such 'leakiness' may be due to a slight ability of the mutant to convert sulphate to cysteine. Many of the 'leaky' mutants gave a negative auxanographic test for growth on sulphate, while showing some response in the spore germination tests.

Table 5. *Incompleteness of the blocked reaction in the mutants*

Growth ability was tested by measurement of colony diameter after growth on a minimal medium with 100  $\mu\text{g./ml.}$  sulphate as the sulphur source for 88 hr. at 37°, and by estimating spore germination in a sulphate-containing liquid medium.

Mutant no.	Colony diam. (mm.)	Degree of germination (%)
1	0	0.4
2	17	6.2
3	18	4.7
6	22	39.1
7	2	5.1
8	5	5.6
Wild-type	65	93.8

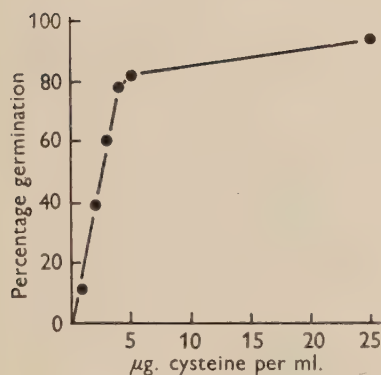


Fig. 1.

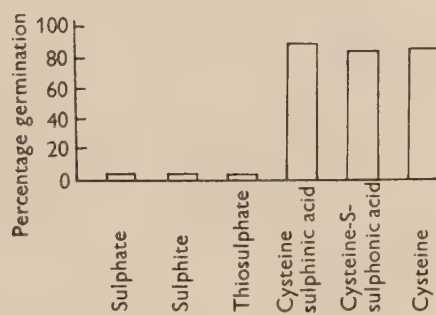


Fig. 2.

Fig. 1. Response of mutant no. 7 to cysteine. The percentage germination of conidia incubated in liquid medium containing cysteine was recorded after 6 hr. incubation at 37°.

Fig. 2. Response of mutant no. 7 to various sulphur sources. Sulphur compounds were added to liquid medium at a concentration of 100  $\mu\text{g./ml.}$  and the percentage germination estimated after incubation at 37° for 6 hr.

## DISCUSSION

The evidence presented suggests that inorganic sulphur compounds may be synthesized biologically into cysteine via at least two different pathways in *Aspergillus nidulans*.

The pathway proposed by Kearney & Singer (1952) for the metabolism of cysteine sulphinic acid in *Proteus vulgaris* and shown to be reversible in rabbit kidney by Chapeville & Fromageot (1954) suggests the pathway starting with sulphate shown in Fig. 3. The latter part of this pathway is taken from the suggestion of Medes & Floyd (1942) that cysteine is oxidized via cysteine sulphenic acid to cysteine sulphinic acid. This would explain the experimental data obtained with the wild-type *Aspergillus nidulans* preparations which use sulphate and sulphite as the inorganic sulphur sources, and also the growth



behaviour of mutants 1, 2, 3, 4, 6, 8 and 9. This pathway, however, provides no explanation for the metabolism of thiosulphate.

The stimulation of cysteine synthesis by serine, when thiosulphate is the inorganic sulphur source, and the complete lack of any stimulating effect of glutamic acid, keto acids or pyridoxal on this system, together with the evidence that there is no intermediate production of cysteine sulphinic acid,

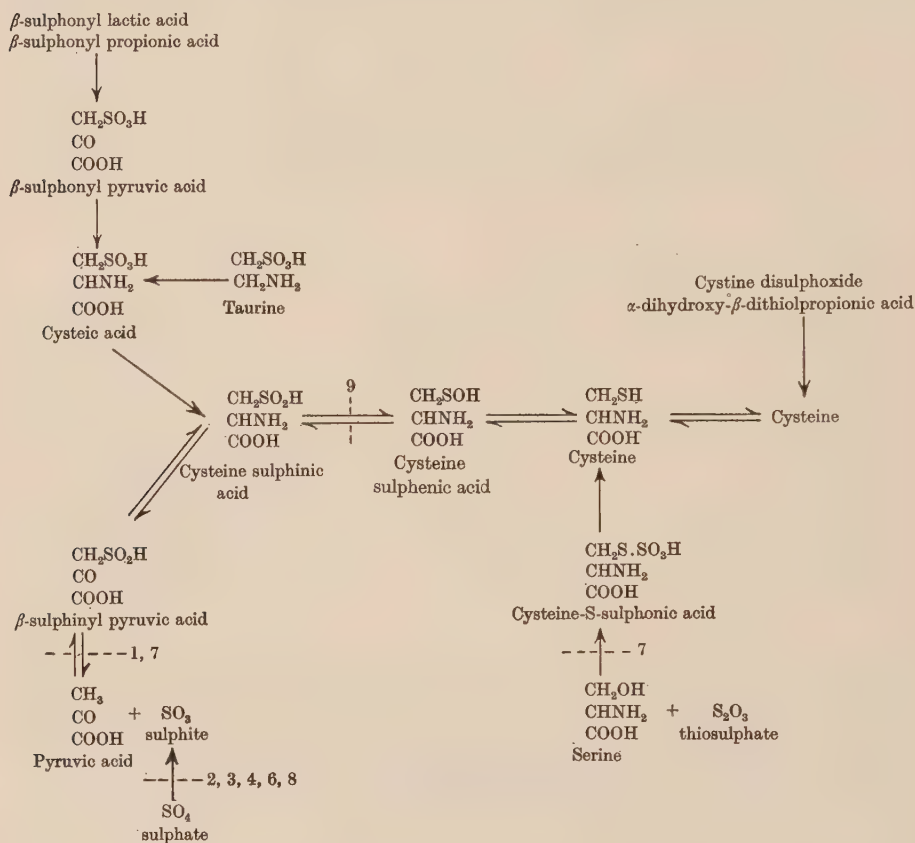


Fig. 3. Scheme for cysteine synthesis in *Aspergillus nidulans*. The positions of the postulated metabolic blocks in the various mutants is shown.

leads to the conclusion that thiosulphate is metabolized by an alternative pathway. The suggestion by Hockenhuil (1949) that thiosulphate and serine combine to give cysteine-S-sulphonic acid, which gives rise to cysteine, would provide an alternative pathway for the metabolism of thiosulphate which would agree with the experimental data.

The pathway in mutant no. 7 is not entirely clear, but a block in the production of some unknown cofactor or energy-providing system common to both pathways, or a permeability change in the mycelium, are possible explanations of the behaviour of this mutant, which is apparently blocked in both metabolic pathways simultaneously.

The experimental data for mutants nos. 1, 6 and 9 do not eliminate the possibility that sulphonylic acid is an intermediate between sulphite and  $\beta$ -sulphonyl pyruvic acid, but the fact that the mutants 'delta', 'eta' and 'lambda' described by Hockenhull (1949) grew on sulphite and cysteine sulphinic acid, but not on sulphonylate, precludes this hypothesis.

The evidence for the intermediacy of cysteine sulphinic acid in sulphate and sulphite metabolism, but not in thiosulphate metabolism, does not support the scheme proposed by Hockenhull (1949), but the proposed scheme in Fig. 3 is entirely compatible with his experimental results. Thus the mutants 'gamma, delta, eta, iota, lambda, sigma and upsilon' of Hockenhull are comparable with mutant no. 6, the mutant 'mu' is equivalent to mutant no. 1 and mutants 'alpha, beta, epsilon, zeta, theta, kappa, xi, nu and omicron' are comparable with mutant no. 9.

The growth behaviour of mutants 1, 6 and 9 indicates that cysteic acid and taurine enter the system in the position proposed by Kearney & Singer (1952), and it is presumed on further evidence from these mutants and from chemical considerations, that  $\beta$ -sulphonyl lactic and  $\beta$ -sulphonyl propionic acids enter the system through cysteic acid. It might be postulated that isethionic acid enters the system by amination to taurine, but the evidence from the mutants indicates that this compound enters the system at the level of sulphite.

Hockenhull produced evidence that several genes are required for the reduction step sulphate  $\rightarrow$  sulphite, which implies that this apparently simple reduction may be complex, there being an unknown number of possible intermediates between sulphate and sulphite. If it be assumed that sulphonylate enters the system after sulphate but before sulphite, possibly by giving rise to one of the postulated intermediates, the results of the growth of the various mutants on sulphonylate may be explained. Alternatively, the equivalence of sodium formaldehyde sulphonylate and free sulphonylic acid may be questioned.

While the metabolic pathways outlined in Fig. 3 adequately explain the experimental data obtained with *Aspergillus nidulans* and with *Escherichia coli*, lack of sufficient evidence does not warrant the extension of this hypothesis to the results obtained with other organisms.

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## The Succinoxidase System of Killer and Sensitive Stocks of *Paramecium aurelia*, Variety 4

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**SUMMARY:** Different pathways of terminal oxidation exist in killer and sensitive stocks of *Paramecium aurelia*, variety 4, stock 51.7. The killers contain an active succinoxidase system which is absent, or present only in small concentrations, in the sensitives. The succinoxidase system might therefore either be necessary for the maintenance of the cytoplasmic particle 'kappa' or is intrinsically associated with it.

'Killer' and 'sensitive' phenotypes of *Paramecium aurelia*, variety 4, occur within stock 51.7 of this organism (Sonneborn, 1947). The two types are identical with respect to the nuclear genotype, but differ by the presence of the cytoplasmic factor, 'kappa', in the killers. The presence of kappa results in an altered respiratory metabolism in the killer cell (Simonsen & van Wagtendonk, 1949, 1952). The respiratory rate of the killer cells is almost double that of the sensitives. The lack of respiratory inhibition in the killers by concentrations of sodium azide, which are strongly inhibitory to respiration in the sensitives as well as the observed low cytochrome oxidase activity, led to the conclusion that the extra respiratory capacity of the killers was not attributable to a cytochrome type respiration. These studies indicated that some basic difference must exist in the terminal respiratory pathways of the two stocks. This must be due to the presence of kappa in the killer cell. Killer and sensitive stocks also differ in their nutritional requirements. An axenic medium which will support the growth of sensitive paramecia will not support the growth of killers (van Wagtendonk, Conner, Miller & Rao, 1953). Further attempts to determine the function of kappa in the metabolism of the killer organisms are described in this communication.

### METHODS

*The stocks of Paramecium aurelia.* Sensitive and killer cultures of *Paramecium aurelia*, variety 4, stock 51.7 were used. The cultures were grown at 27° according to methods previously described for large mass cultures (Simonsen & van Wagtendonk, 1952).

*Preparation of the homogenates.* Approximately 6 l. of the culture which had been fed with *Aerobacter aerogenes* 48 hr. previously were concentrated to a volume of 20 ml. by filtration on a Berkefeld filter. This concentrated suspension of paramecia was then transferred to an electromigration apparatus in order to effect a further concentration and washing of the cells (van Wagtendonk, Simonsen & Zill, 1952). A modified migration medium was made up as follows: 200 mg. NaCl; 50 mg. KCl; 100 mg. CaCl<sub>2</sub>·H<sub>2</sub>O; 50 mg. MgSO<sub>4</sub>·7H<sub>2</sub>O;

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25 mg.  $K_2HPO_4$ ; 25 mg.  $KH_2PO_4$ , dissolved in 1 l. glass-redistilled water. The concentrated suspension of paramecia (8–10 ml.) was withdrawn from the apparatus and transferred to a 10 ml. graduated conical centrifuge tube. The total number of paramecia was determined by diluting measured samples of the suspension to appropriate volumes and counting the number of organisms present directly. At least three determinations were made on each suspension. The suspension was then centrifuged at 1500 r.p.m. in a refrigerated centrifuge, the supernatant fluid poured off without special precautions, and the loss in centrifugation determined by a direct count of the organisms present in the supernatant fluid. The organisms were resuspended in the desired respiration medium and the concentration was adjusted to about 500,000 organisms/ml. This suspension was then transferred to a tight-fitting Potter-Elvehjem glass homogenizer powered by a laboratory stirring motor. Each sample was homogenized for 4 min. and then examined for the presence of whole organisms, which were removed with a micropipette. All operations involved in the preparation of the homogenates were carried out in a cold room at 4°, and all solutions and glassware were chilled before use.

*Media used for homogenization.* Three different media were used in preparing the homogenates. In one series of experiments the saline phosphate solution used for the electromigration of the paramecia was used. In another series distilled water was added to the packed organisms before homogenization. In a third series, sodium phosphate buffer (pH 7·2) was added so that the final concentration of phosphate was 0·05 M. The pH value of all solutions was maintained at 7·1–7·2.

*Respiration measurements.* The Cartesian diver technique was used for these determinations (Simonsen & van Wagtendonk, 1952). The main drop contained homogenate equivalent to 1500–1800 paramecia. It was determined that a linear endogenous respiratory response proportional to the numbers of organisms used could be obtained when the equivalent of 1000–5000 paramecia were used for each diver vessel. Inhibitors and substrates were added according to the needs of the particular experiment by the side-drop technique. Following a 30 min. equilibration period, the side drops were added to the homogenates and readings were made at 10 min. intervals for 60 or 90 min. thereafter. Each experiment was repeated from three to five times with appropriate controls. The tables represent a composite of the results obtained. There was little variation in the relative rates measured in duplicate experiments.

*Substrates and inhibitors.* Sodium succinate alone, or in combination with other substances, was added to the homogenates to a final concentration of 0·05 M. Methylene blue was used at a concentration of  $5 \times 10^{-4}$  M. Following the suggestions of Schneider & Potter (1943), Swingle, Axelrod & Elvehjem (1942) and Keilin & Hartree (1949), aluminium chloride and calcium chloride were incorporated in the medium at a final concentration of  $10^{-4}$  M. Sodium malonate, 0·08 M, was used as a specific inhibitor of the succinic dehydrogenase system. The concentrations of all of these solutions before addition to the homogenate were ten times the final concentrations reported above. The pH value was adjusted to 7·2.

## RESULTS

*Influence of homogenization media upon endogenous respiratory rates*

The measured succinoxidase activity of tissue homogenates is dependent upon the method of preparation of the homogenates (Keilin & Hartree, 1949). Factors which influence the activity include salt concentration, nature of the buffer system used, and the concentration of phosphate in the medium. Most of the effects observed were attributable to changes in the colloidal nature of the enzyme complexes comprising the particular preparation.

Homogenates of killers and sensitives prepared in the migration medium were compared with respect to their endogenous respiratory rates. It was found that the rate for the killer preparation was  $11.5 \times 10^{-6}$   $\mu\text{l.}/\text{organism}/\text{hr.}$  compared with  $5.5 \times 10^{-6}$   $\mu\text{l.}/\text{organism}/\text{hr.}$  for the sensitives (Table 1). These results confirm the earlier observations made with intact organisms, that the killers have a respiration rate double that of the sensitives.

Table 1. *Influence of medium on oxygen consumption of homogenates of killer and sensitive paramecia*

Medium	Sensitive		Killer	
	$q_{\text{O}_2}$ *	$\Delta q_{\text{O}_2}$ †	$q_{\text{O}_2}$	$\Delta q_{\text{O}_2}$
Saline solution	5.5	—	11.5	—
+ succinate (0.05 M)	6.0	0.5	12.8	1.3
Distilled water	2.8	—	11.8	—
+ succinate (0.05 M)	3.4	0.6	6.0	- 5.8
Phosphate (0.05 M)	0.4	—	11.7	—
+ succinate (0.05 M)	0.7	0.3	26.5	14.8

\* The  $q_{\text{O}_2}$  is expressed as  $\mu\text{l.} \times 10^{-6}$  of oxygen consumed/cell-hour.

† The  $\Delta q_{\text{O}_2}$  represents the difference between the endogenous rate and that measured with added substrate.

The figures in the table represent the average of four separate experiments with a  $\pm 10\%$  deviation from the mean.

When distilled water or phosphate buffer (0.05 M) were used, the endogenous  $q_{\text{O}_2}$  ( $\mu\text{l. O}_2$  consumed/organism/hr.) of the homogenate of killer organisms remained at the same high level (about  $12 \times 10^{-6}$   $\mu\text{l.}/\text{organism}/\text{hr.}$ ) (Table 1). By contrast, the homogenate of sensitive organisms exhibited a lowered  $q_{\text{O}_2}$  when distilled water was used (about  $2.8 \times 10^{-6}$   $\mu\text{l.}/\text{organism}/\text{hr.}$ ). When phosphate buffer was used the  $q_{\text{O}_2}$  was diminished to a value which was below the sensitivity of the measuring apparatus. From these results it may be concluded that the endogenous respiration of the killer homogenates is independent of the method of preparation, whereas that of the sensitives is strictly dependent.

*Influence of added succinate.* The addition of succinate (0.05 M) to homogenates of sensitive organisms prepared in any of the three media caused only a slight stimulation of the endogenous  $q_{\text{O}_2}$ . With the killer preparations, however, the effects of added succinate were pronounced and dependent upon the media used for homogenization. When saline solution was used, there was a slight stimulation of the  $q_{\text{O}_2}$  by added succinate which was comparable to



that found with sensitive organisms prepared in the same medium (Table 1). Distilled water preparations of killer organisms responded to the addition of succinate with a marked inhibition in respiration (Table 1). Finally, in the phosphate buffer medium, the added succinate caused a doubling of the respiratory rate of the killer preparation (Table 1). These data provide evidence for the presence of a succinoxidase system in the killers which is dependent upon the presence of phosphate in the homogenization medium. In other succinoxidase systems, increased activity in the presence of phosphate has been noted (Keilin & Hartree, 1949; Ball & Cooper, 1949; Slater, 1949; Bonner, 1954). It was concluded that this effect was due to the colloidal state of the preparations as influenced by phosphate. Esterification of inorganic phosphate was demonstrated when succinate was oxidized either aerobically or anaerobically by cell-free extracts of *Escherichia coli* (Hersey & Ajl, 1951*a, b*). Also it has been shown that phosphate utilization is dependent upon the electron acceptor system present (Kearny, Singer & Zastrow 1955). Thus, phosphate may act in the killer preparation by influencing the colloidal nature of the succinoxidase system. Since there was only little stimulation by added succinate in the homogenates of sensitive organisms and since there was no stimulation in the presence of phosphate, it may be concluded that succinoxidase is absent, or has a different activity in the sensitive system. The magnitude of the differences between killer and sensitive organisms when phosphate buffer was used made this the medium of choice for further experiments on the succinoxidase system.

*Effects of the addition of methylene blue* (0.0005 M). Further, to characterize the activity of succinate, methylene blue was added to homogenates prepared in phosphate buffer. Methylene blue in the presence of calcium and aluminium ions, but without added succinate, caused a marked stimulation of the respiration of both killer and sensitive homogenates (Table 2). The increase was of the same order of magnitude in both preparations,  $7.3 \times 10^{-6}$   $\mu\text{l.}/\text{organism}/\text{hr.}$  for the killers and  $8.8 \times 10^{-6}$  for the sensitives. This stimulation of the endogenous metabolism by methylene blue indicated the presence of some system common to both killers and sensitives and independent of the presence or absence of kappa. Further, the presence of malonate (0.08 M) did not influence this methylene blue catalysed oxidation process (Table 3). Therefore this stimulation cannot be due to Krebs-cycle activity and must be due to the oxidation of some substrate present in high concentration in both cell types. These results may be contrasted to those found with *Paramecium caudatum* in which the addition of methylene blue to homogenates resulted in no increase in the endogenous respiratory rate (Humphrey & Humphrey, 1947, 1948).

*Effect of succinate in the presence of methylene blue.* The addition of succinate to the homogenates of sensitives stimulated by methylene blue caused only a slight additional increase in the  $q_{O_2}$  (Table 2). With the homogenates of killers, the addition of succinate with methylene blue stimulated the oxygen consumption to a  $q_{O_2}$  of  $24.5 \times 10^{-6}$   $\mu\text{l.}/\text{organism}/\text{hr.}$  The actual increase caused by the addition of succinate in this system was comparable to the increase over the endogenous rate produced by succinate in the absence of methylene blue.

Therefore the succinic dehydrogenase activity of the killers is independent of the presence or absence of methylene blue.

*The effect of malonate.* Malonate when added to homogenates of killers as well as sensitives in the presence or absence of methylene blue had little effect upon the respiratory rates (Table 3). However, when malonate was added to the killer homogenate in the presence of succinate, the stimulation which had been noted with succinate was almost completely abolished. The use of malonate as a competitive inhibitor of succinate oxidation demonstrates the true nature of the succinoxidase activity which was measured in the killer homogenates.

Table 2. *Metabolism of succinate in the presence and absence of methylene blue*

Substrate	Sensitive		Killer	
	$q_{O_2}^*$	$\Delta q_{O_2}^\dagger$	$q_{O_2}$	$\Delta q_{O_2}$
Endogenous (phosphate, 0.05 M)	0.5	—	11.7	—
Methylene blue	9.3	8.8	19.0	7.3
Succinate (0.05 M)	0.8	0.3	24.5	12.8
Methylene blue + succinate (0.05 M)	10.5	10.0	31.0	19.3

\* The  $q_{O_2}$  is expressed as  $\mu\text{l.} \times 10^{-6}$  of oxygen consumed/cell-hour.

† The  $\Delta q_{O_2}$  represents the difference between the endogenous rate and that measured with added substrate.

The figures in the table represent the average of four experiments with a  $\pm 10\%$  deviation from the mean.

Table 3. *Influence of added malonate on the succinate stimulated respiration*

Substrate	Sensitive		Killer	
	$q_{O_2}^*$	$\Delta q_{O_2}^\dagger$	$q_{O_2}$	$\Delta q_{O_2}$
Endogenous (phosphate, 0.05 M)	1.0	—	11.2	—
Methylene blue	10.3	9.3	19.2	8.0
Methylene blue + succinate (0.05 M)	11.7	10.7	33.2	22.0
Methylene blue + malonate (0.08 M)	10.7	9.7	17.2	6.0
Methylene blue + succinate (0.05 M) + malonate (0.08 M)	10.9	9.9	21.0	9.8

\* The  $q_{O_2}$  is expressed as  $\mu\text{l.} \times 10^{-6}$  of oxygen consumed/cell-hour.

† The  $\Delta q_{O_2}$  represents the difference between the endogenous rate and that measured with added substrate.

The figures in the table represent the average of four separate experiments with a  $\pm 10\%$  deviation from the mean.

## DISCUSSION

The results of the present study provide further evidence for the existence of different pathways of terminal oxidation as between killer and sensitive stocks of *Paramecium aurelia*, variety 4, stock 51.7. The demonstration of an active succinoxidase system in the killers which is absent, or present in only low concentration, in the sensitives indicates that the kappa particles possess a metabolic function in the cell. An additional difference between the two types has been established with regard to the endogenous metabolism of cell homogenates.

Evidence for the presence of succinic dehydrogenase in other species of paramecia as well as in other ciliated protozoa has been compiled. The early work of Leichsenring (1925) demonstrated that the addition of succinate to *Paramecium caudatum* resulted in a stimulation of respiration of between 8 and 9%. Humphrey & Humphrey (1947, 1948), using homogenates of *P. caudatum*, noted an increase over the endogenous respiration of 125% in the presence of succinate which could be reversed by the addition of malonate. Later work (Holland & Humphrey, 1953*a, b*; van Grembergen & Reynaerts-de Pont, 1952) with intact *P. caudatum* confirmed the results of Leichsenring. Seaman (1949, 1950, 1951, 1952) compiled evidence for the existence of the Krebs cycle in *Tetrahymena pyriformis* (*S*); but with *T. pyriformis* (*GL*), Ryley (1952) found no evidence for the oxidation of succinate in the presence or absence of added cytochrome *c*, although an active dehydrogenase was demonstrated by the Thunberg technique.

In all of the work cited, the magnitude of the succinic dehydrogenase or oxidase activity was slight compared with the activities in mammalian tissues; this has led to the suggestion that this system may be relatively unimportant to the economy of the paramecia when compared to other pathways (Holland & Humphrey, 1953*b*).

In the case of stock 51.7 of *Paramecium aurelia*, the genetic transformation from killer to sensitive, in so far as can be determined, involves only the loss of kappa particles. This loss, however, results in the elimination of the ability to oxidize succinate. Under the cultural conditions used, the sensitive organisms grow at the same rate as the killers. From this it may be concluded that succinoxidase is either necessary for the maintenance of the kappa particles or is intrinsically associated with them. When kappa is eliminated, i.e. in the sensitives, succinoxidase is no longer important to the economy of the organism.

An analogy may be made between this system and the acriflavine-induced 'small colony' mutants in the yeast *Saccharomyces cerevisiae* (Ephrussi, l'Heritier & Hottingeur, 1949; Slonimski & Ephrussi, 1949; Ephrussi & Hottingeur, 1951). The induction of 'small colony' mutants involves the loss of a cytoplasmic particle necessary for the synthesis of cytochrome oxidase and succinoxidase. Hirsch (1952) has established that this transformation involves a modification in the enzymic make-up of the organism with the loss of the enzymes cytochrome oxidase, succinic dehydrogenase,  $\alpha$ -glycerophosphate dehydrogenase, and a DPN-cytochrome *c* reductase, all of which are linked to the particulate component of normal yeast.

In *Paramecium aurelia* the loss of kappa results in the loss of succinoxidase as well as some complex of enzymes which contribute to the high endogenous metabolism of the killers. The demonstration of a higher cytochrome oxidase activity in the sensitives, determined by the method of Smith & Stotz (1949), would prevent a strict analogy. The close association of succinoxidase and cytochrome oxidase as found in many systems is not obligatory for all organisms. In the filarial parasites there is ample evidence for the presence of succinic dehydrogenase activity in association with a particulate fraction,



while there is no evidence for the presence of cytochrome oxidase activity (Bueding, 1949; Bueding & Charms, 1951; Ross & Bueding, 1950).

The hypothesis that the enzymic activities of succinoxidase and other enzymes which catalyse terminal oxidative processes are associated directly with kappa particles, is attractive. However, Preer & Stark (1953) with purified preparations of kappa particles were unable to demonstrate any enzymic activity in their preparation, but they recognized the limitations of the histochemical techniques used. If these enzymes are not an intrinsic part of the kappa particle, then they must be necessary for the production of energy for the maintenance and reproduction of the kappa particle and the synthesis of the killer substance, paramecin.

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## An Analysis of the Process of Adaptation of Influenza Virus B of Recent Human Origin to the Mouse Lung

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**SUMMARY:** During the course of adaptation to the mouse lung of the Rob strain of influenza B, 'pure' clones of virus were isolated from bronchial washings at succeeding passages by the limiting infective dilution technique in chick embryos. The clones were studied for various *in vitro* and *in vivo* properties. A progressive dominance was obtained in the virus population of virus particles causing extensive pulmonary consolidation. Those particles which produced extensive consolidation became resistant to inhibition of heated virus haemagglutinin by sheep mucin. A complete correlation was found between the appearance of resistance to sheep mucin inhibitor and a decrease of enzymic activity on sheep mucin.

From the heterogeneous population of particles found in the early course of mouse passage a virus was isolated which exhibited all of the tested properties of the adapted virus. In comparison to a non-pathogenic virus form, these properties included: (a) the production of extensive pulmonary consolidation, high mortality of mice, and multiplication of virus on serial mouse passage; (b) the production of a more rapid growth rate, and the ability to multiply to a greater extent; (c) the inability of heated virus haemagglutinin to be inhibited by sheep mucin and ovomucin; (d) a decrease of enzymic activity on sheep mucin and mouse lung inhibitors under certain experimental conditions; and (e) a high position in the fowl red cell receptor gradient.

The above data offer presumptive evidence that one of the processes operative in the adaptation process of Rob virus to the mouse lung is a selection of mutants found in the unadapted heterogeneous virus population.

Studies dealing with the adaptation of influenza viruses to the mouse lung have revealed that a number of properties may differentiate mouse-adapted from unadapted strains. These properties include differences in multiplication characteristics in mice (Wang, 1948; Davenport & Francis, 1951; Briody & Cassel, 1955) and eggs (Ginsberg, 1953), in reactivity to the  $\beta$  inhibitor (Davenport, 1952; Briody, Cassel & Medill, 1955), and in other *in vitro* characteristics such as heat stability (Burnet, 1951*a*; Ledinko, 1955), inhibition of indicator virus haemagglutination by mucoids (Ledinko, 1955), and position in the receptor gradient (Burnet, 1951*a*; Ledinko, 1955). These findings indicate that changes in the virus population may play an important part during the course of adaptation.

By studying the distribution of mouse lung pathogenicity among 'pure' clone samples of the CAM strain of influenza A prime virus at various stages of adaptation, Burnet & Lind (1954) concluded that the results obtained supported the hypothesis of a successive replacement of one population by another

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more suited for survival. This would agree with the findings of Davenport (1954) that not all virus particles in an unadapted virus population possess an equal potential to adapt to the mouse lung. Briody & Cassel (1955) and Briody *et al.* (1955) have obtained evidence which indicates that the process of adaptation is accompanied by a series of interrelated changes in the virus population.

In a previous study with the Rob strain of influenza B virus (Ledinko, 1955) it was found that mouse-adapted Rob virus differed from its unadapted parent in a number of *in vitro* as well as *in vivo* characteristics. Therefore a study was undertaken of various *in vitro* as well as *in vivo* properties of 'pure' clones of Rob virus, isolated by the limiting infective dilution technique in chick embryos at succeeding mouse passages, in an attempt to elucidate some of the characteristics of the survival advantage and consequent adaptation to the mouse lung of this virus.

#### METHODS

*Virus.* The Rob strain of influenza B virus was used. This strain had received eleven limit infective dilution passages allantoically in 11-day embryonated eggs since its initial isolation in the amnion. The complete history has been described (Ledinko & Perry, 1955). A CO<sub>2</sub> ice cabinet was used for storage of all virus preparations.

*Limit infective dilution (LD) technique.* All LD isolations refer to fluids obtained from an inoculum of the test material which did not infect more than 40 % of 11-day embryonated eggs, using 15–20 eggs per inoculum. The eggs were inoculated allantoically, and allantoic fluids harvested after 3 days' incubation.

*Haemagglutinin (HA) titrations.* To 0.25 ml. of each two-fold serial dilution of the test material, 0.25 ml. of a 1 % suspension of fowl erythrocytes was added. The tests were read as soon as the cells in the control tubes had settled at room temperature. The end-point was taken as the + degree of partial agglutination. The titre is expressed as the reciprocal of the dilution at the end-point. The dilution at the end-point contains one fowl agglutinating dose of virus (FAD).

*Infectivity titrations.* Tenfold serial dilutions of the test material were inoculated allantoically into groups of 10 to 12-day chick embryos. 0.05 ml. of each dilution was inoculated into six eggs. After incubation for 72 hr. at 35°, the eggs were tested for HA. On this basis, the 50 % egg infectivity end point (EID<sub>50</sub>) was calculated according to Reed & Muench.

*Inoculation of mice.* 0.05 ml. of the test material diluted to 1 FAD was inoculated intranasally under light chloroform-ether anaesthesia into groups of 4- to 5-week old mice. For virus passage, lungs were removed from six mice 2 to 3 days after inoculation, and bronchial washings were collected according to a technique described by Fazekas de St Groth (1948), and used previously with Rob virus (Ledinko & Perry, 1955). The individual washings were pooled, diluted to 1 FAD, and inoculated into mice, usually on the same day.

To determine the mouse pathogenicity score, using six mice per group, mice still alive 7 days after inoculation were killed and examined for lung lesions. The method of scoring lung lesions was as follows:

- 5·0 Complete consolidation, death of mouse on 4th or 5th day.
- 4·0 Complete consolidation, death of mouse on 6th or 7th day.
- 3·0 More than half of lung consolidated.
- 2·0 Approximately half the visible area of the lung surface consolidated.
- 1·0 About 1/6 to 1/4 of lung surface consolidated.
- 0·5, 0·2, and trace for smaller areas of damage.

*Inhibitors.* Sheep salivary gland mucin was prepared from submandibular glands according to McCrea (1951). The titres refer to a stock solution containing 1 % (dry wt.) in normal saline.

*Cyst mucin* purified from the contents of an ovarian cyst by the method of McCrea (1949) was used. The stock solution contained 1 % of dried material in normal saline.

*Ovomucin* was prepared as described by Gottschalk & Lind (1949). The titres refer to a stock solution containing 0·2 % (dry wt.) in normal saline.

*Meconium* was prepared according to the technique described by Curtain, French & Pye (1953). The titres refer to a stock solution containing 1 % (dry wt.) in normal saline.

*Mouse lung suspension* was prepared from 4-week old mice. Lungs were removed from twelve normal mice, ground with alundum, and suspended in normal saline (1 ml./lung). The suspension was lightly centrifuged, the supernatant fluid removed and stored at 4°.

*Bronchial washings* were collected from twelve normal mice by the technique of Fazekas de St Groth (1948). The washings were pooled and stored at 4°.

*Production of indicator virus.* Virus was diluted with 1 vol. of citrate-borate saline (10 % by volume of standard borate buffer at pH 8·5 and 0·2 % sodium citrate in normal saline) and heated at 56° for 30 min. (Stone, 1949).

*Titration of inhibitor-sensitivity of virus.* See Stone (1949). Serial two-fold dilutions of inhibitor were prepared in 0·25 ml. normal saline. An equal volume of a dilution of indicator virus containing 5 FAD was added to each tube. After an incubation of 30 min. at room temperature, 0·25 ml. of a 1 % suspension of red cells was added. The test was read by the pattern of HA after a further hour at room temperature. The titre is expressed as the reciprocal of the dilution of the inhibitor at the end point of partial agglutination.

*Titration of inhibitor-destroying activity of virus.* See Stone (1949). Serial two-fold dilutions of virus were prepared in 0·25 ml. volumes of calcium-acetate saline, and to each was added 0·25 ml. of a standard dilution of inhibitor (containing five to ten inhibitory doses, each dose being capable of inhibiting 5 FAD of the indicator virus used). After incubation for 1 hr. at 37° the mixtures were heated at 65° for 30 min. to destroy the virus haemagglutinin, then cooled to room temperature. One drop of appropriate indicator virus containing 5 FAD was added to each tube, and after 30 min. at room temperature 0·25 ml. of a 1 % suspension of fowl cells. The test was read by the pattern of HA after a further hour at room temperature. The

inhibitor-destroying (ID) titre is expressed as the reciprocal of the dilution of virus at the end-point of partial agglutination.

*Position in the receptor gradient.* The method used was described by Isaacs & Edney (1950). Semi-purified receptor-destroying enzyme (RDE) of *Vibrio cholerae* prepared according to the method of Ada & French (1950) was used. Fowl cells were treated for 30 min. at 37° with graded dilutions of RDE and subsequently washed with citrate saline. To 0.25 ml. of a 1 % suspension of treated cells, 0.25 ml. of 5 FAD of virus was added. The titre is read as the reciprocal of the dilution of RDE which results in cells agglutinated to a + level of partial agglutination.

#### EXPERIMENTAL

*Mouse lung pathogenicity of LD fluids isolated at each succeeding mouse passage.* The course of adaptation to the mouse lung of Rob virus, using 1 FAD as the inoculum at each passage, is shown in Fig. 1. Inoculation of the virus resulted in the production of extensive pulmonary consolidation, followed by a decrease in consolidation with continued passage, and a subsequent gradual rise in pulmonary lesions. This biphasic course is similar to that obtained using Rob virus which had undergone three LD allantoic passages, as

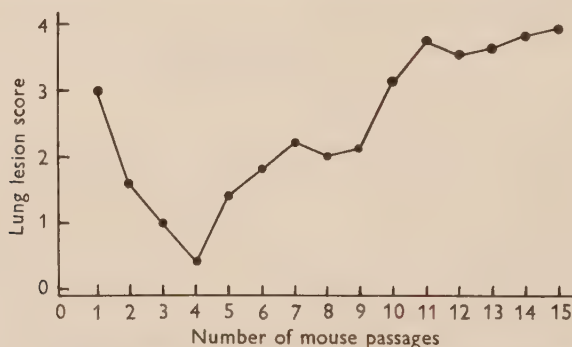


Fig. 1. Mouse lung pathogenicity scores obtained with Rob virus on serial mouse passage.

described previously (Ledinko & Perry, 1955). It was considered then that the first phase might be the result of virus particles whose properties were related to the previous (natural) human passage, since continued passage in the chick embryo resulted in a gradually diminishing ability of the virus to produce consolidation of the mouse lung in the early course of mouse passage. Therefore, in the present study, the course of 'true' adaptation will be considered to begin with the 4th mouse passage. Beginning with this passage, at which minimal lung lesions were seen, a gradual increase in the extent of pulmonary consolidation was observed. Mortality and extensive pulmonary consolidation were obtained by the 11th passage and were transferable through a number of additional passages.

The LD fluids isolated in eggs from the bronchial washings obtained at each mouse passage were tested for mouse lung pathogenicity. Only fluids which



were subsequently shown to be 'pure' after an additional limit dilution passage (as indicated by the sheep mucin inhibitor-sensitivity property described in the next section) are included in the data. One FAD of each fluid was inoculated. As may be seen in Table 1, a progressive change was noted in the virus population leading to a progressive dominance of those virus particles which produced extensive pulmonary consolidation. Similar results were obtained by Burnet & Lind (1954) using the CAM strain of influenza A prime virus.

Table 1. *Mouse lung pathogenicity of LD fluids isolated at each mouse passage*

Mouse passage no.	Average lesion score	No. of LD fluids	No. of fluids. Pathogenicity score (inoculum 1 FAD)				
			Class				
			0	I	II	III	IV
2	1.6	6	0	2	4	0	0
3	1.0	12	0	1	8	3	0
4	0.4	10	0	6	2	2	0
5	1.4	10	0	1	3	4	2*
6	1.8	17	0	3	3	7 (5*)	4*
7	2.2	12	0	0	1	3 (1*)	8*
8	2.0	17	0	0	0	1*	16*
9	2.1	13	0	0	0	0	13*
11	3.7	4	0	0	0	0	4*
15	3.9	5	0	0	0	0	5*

\* Non-indicators with sheep mucin inhibitor.

*Sheep mucin inhibitor-sensitivity titrations of LD fluids isolated at each mouse passage.* In a previous study (Ledinko, 1955) of the differential characteristics between mouse-unadapted Rob virus and its mouse-adapted derivative, it was found that one of the differentiating properties was the conversion of unadapted virus to the indicator phase, by heating at 56° for 30 min., as tested with sheep mucin inhibitor. The adapted virus was not converted; i.e. no inhibition of indicator virus haemagglutination by sheep mucin occurred. It was similarly found in the present study that the adapted virus could not be converted to the indicator phase even after heating with citrate-borate buffer at pH 8.5 (Stone, 1949). Therefore, the LD fluids isolated at each mouse passage were tested for their ability to be inhibited after heating by sheep mucin inhibitor. Those LD fluids which could not be converted into indicators are marked with an asterisk in Table 1, while the sheep mucin inhibitor-sensitivity titres of the fluids are given in Table 2.

It can be seen that the ability to be converted to the indicator state was lost concomitantly with a rise in pulmonary consolidation during mouse passage, as indicated by the average lesion score. Furthermore, only those fluids which produce extensive pulmonary consolidation lost the indicator property. In Table 2, in contrast to the gradual changes in virulence of the virus population, a sharp differentiation in inhibitor titres is seen. This may be an indication of a single-step mutational change in contrast to the multiple-step change shown

to exist for mouse lung virulence (Burnet, 1954). In a similar study by Briody *et al.* (1955), using strains of type A prime and type B influenza viruses, the appearance of extensive pulmonary consolidation was correlated with the sudden loss of susceptibility by unheated virus to both HA-inhibition and to neutralization by B inhibitor in normal ox serum. In the present study the haemagglutination by active unadapted and adapted virus was not inhibited by sheep mucin.

Table 2. *Sheep mucin inhibitor sensitivity titrations of LD fluids*

Mouse passage no.	Average lesion score	No. of LD fluids	Sheep mucin inhi- bitor sensitivity test
			Range of log of inhibitor titre*
2	1.6	6	3.8-3.9
3	1.0	12	3.8-4.0
4	0.4	10	3.8-3.9
5	1.4	10	3.8-4.0 (8); <1-1.1 (2)†
6	1.8	17	3.9-4.0 (8); <1-1.1 (9)
7	2.2	12	3.9-4.0 (3); <1-1.2 (9)
8	2.0	17	<1-1.2
9	2.1	13	<1-1.1
11	3.7	4	<1-1.2
15	3.9	5	<1-1.2

\* Increasing the time of contact between inhibitor and indicator virus before adding red cells to 3 hr. incubation did not alter the titre obtained.

† Number in parentheses represents number of LD fluids showing the titre indicated.

*The sheep mucin inhibitor-destroying activity of LD fluids isolated at each mouse passage.* The inhibitor-destroying activity of virus has been used as a measure of its enzymic activity (Stone, 1949). The titration of this characteristic gives an estimate of the dilution of virus capable of destroying a standard dose of inhibitor under specified conditions. The sheep mucin inhibitor-destroying activity of the 'pure' clones of virus isolated at various mouse passage levels is shown in Table 3. The activity is expressed in terms of a ratio of the inhibitor-destroying titre to the haemagglutinin titre. The results indicate that complete enzymic destruction of sheep mucin inhibitor occurred in the early passages (ratio 0.5-1.0), while no destruction of the inhibitor occurred in the later passages (ratio <0.01), under the experimental conditions used. There is a complete correlation between the marked loss of the indicator property and decrease of enzymic activity of the LD fluids tested.

*Separation of components from mixed virus population.* The experimental results in the preceding sections revealed that a heterogeneous collection of viruses was present in the bronchial washings of the early mouse passages, both in regard to pathogenicity and indicator property. An inspection of the data in Table 1 indicated that at least four different virus components were found with the following differential characteristics: (1) non-pathogenic, indicator, NP-I (5); (2) pathogenic, indicator, P-I (5); (3) pathogenic, non-indicator from an early passage, P-NI (5); and (4) pathogenic, non-indicator from a late passage P-NI (15). Viruses of types 1-3 were selected from the 5th mouse

passage, and of type 4 from the 15th passage, and carried through five to six additional egg LD passages. The properties of these separated presumably 'pure' viruses are shown in Table 4. These properties remained constant through the LD passages.

Table 3. *Sheep mucin inhibitor-destroying activity of LD fluids*

Mouse passage no.	No. of LD fluids	Sheep mucin inhi- bitor-destroying test
		Range of ID titre/HA titre*
2	6	0.5-1.0
3	12	0.5-1.0
4	10	0.5-1.0
5	10	0.5-1.0 (8); < 0.01 (2)
6	17	0.5-1.0 (8); < 0.01 (9)
7	12	0.5-1.0 (3); < 0.01 (9)
8	17	< 0.01
9	13	< 0.01
11	4	< 0.01
15	5	< 0.01†

\* ID titre is reciprocal of dilution of virus capable of destroying the inhibitor for indicator NP-I (see next section) in a standard dose of sheep mucin.

† To determine whether longer incubation would enable the adapted virus to act on the sheep mucin inhibitor, incubation was prolonged for 18 hr. at 37°. Some destruction of the inhibitor did occur (ID/HA = approx. 0.1).

Table 4. *Differential characteristics of separated viruses*

Type	No. of LD passages	Mouse lesion score	Log of sheep mucin inhibitor sensitivity titre
NP-I (5)*	5	0.1	3.9
P-I (5)	6	2.8	3.8
P-NI (5)	6	3.7	< 1.0
P-NI (15)	5	3.9	< 1.0

NP = non-pathogenic, P = pathogenic, I = indicator, NI = non-indicator.

\* Number in parentheses indicates the mouse passage from which fluid was isolated.

*Determination of specificity of inhibitor effect.* The inter-relationship of the development of extensive pulmonary consolidation with the loss of conversion to the indicator state as tested with sheep mucin inhibitor was noted previously. This prompted the testing of other inhibitors in inhibitor-sensitivity tests using the following viruses: NP-I (5), P-NI (5) and P-NI (15). The inhibitors tested with the three heated viruses were: sheep mucin, mouse lung, cyst mucin, ovomucin, meconium and bronchial washings. The results are shown in Table 5. It was found that heated NP-I (5) virus was inhibited by all of the inhibitors tested. Little or no effect on heated P-NI (5) and P-NI (15) viruses was shown by ovomucin and sheep mucin inhibitors only. The other inhibitors tested inhibited both the P-NI (5) and P-NI (15) viruses.

In view of the findings of Davenport (1952) that haemagglutination by unadapted influenza virus was inhibited by the inhibitor in normal mouse lung



to a greater degree than was observed with the adapted virus, it might be expected that the above inhibitor-sensitivity tests using the mouse lung inhibitor would also reveal a marked difference between NP-I (5) and P-NI (15) viruses. However, the lung extract would not only contain the cellular receptors concerned in the course of infection. A further experiment was done in order to determine whether a differential effect of the two viruses could be detected in the enzymic test. The mouse lung inhibitor-destroying activity of NP-I (5), P-NI (5) and P-NI (15) viruses was therefore tested. The inhibitor-destroying titre was obtained using three different indicator viruses, as noted in Table 6. Stone (1949) had shown that the type of indicator virus markedly influenced the titre obtained with some strains. As seen in Table 6, NP-I (5)

Table 5. *Determination of specificity of inhibitor effect*

Heated virus	Log of inhibitor sensitivity titre					Bronchial washings
	Sheep mucin	Mouse lung	Cyst mucin	Ovomucin	Meconium	
NP-I (5)	3.9	3.0	3.8	3.1	3.5	1.7
P-NI (5)	<1.0	2.6	3.7	1.2	3.4	1.7
P-NI (15)	<1.0	2.5	3.5	<1.0	3.5	1.6

Table 6. *Mouse lung inhibitor-destroying titrations*

Virus	Mouse lung inhibitor-destroying test. ID titre/HA titre		
	Indicator	Indicator	Indicator
	NP-I (5)	P-NI (15)	P-NI (5)
NP-I (5)	0.6	0.7	0.6
P-NI (5)	<0.01	0.6	0.8
P-NI (15)	<0.01	0.5	0.9

virus completely destroyed the mouse lung inhibitor. P-NI (5) and P-NI (15) viruses were found to be fully active against the mouse lung inhibitor for their own indicators. No activity was manifest by P-NI (5) and P-NI (15) viruses when NP-I (5) virus was used as the indicator virus. Fazekas de St Groth (1950) has shown that the inhibitor of bronchial washings for heated Lee virus is destroyed during influenza virus infection.

*Receptor gradient position of NP-I (5), P-NI (5) and P-NI (15) viruses.* The experimental results shown in the preceding sections revealed that P-NI (5) and P-NI (15) viruses showed the same reactivity with a number of inhibitors (Tables 5 and 6), as well as the capacity to produce extensive pulmonary consolidation (Table 4). The position of NP-I (5), P-NI (5), and P-NI (15) viruses in the receptor gradient using fowl red cells treated with graded amounts of RDE was next examined. The NP-I (5) virus was found to occupy a low position in the gradient, with a titre of 16, while P-NI (5) and P-NI (15) viruses occupied a much higher position in the gradient, both viruses having a titre of 128.

*Mouse lung passage of P-NI (5) and P-NI (15) viruses using bronchial washings.* The adapted P-NI (15) virus produces extensive pulmonary consolidation, mortality of mice, and multiplication of virus as determined by HA and egg infectivity titres of the bronchial washings on serial mouse passage. The behaviour of P-NI (5) virus under similar experimental conditions was compared to that of P-NI (15) virus. One FAD of 42 hr. LD allantoic fluid preparations was inoculated. Bronchial washings collected 2 days after virus inoculation were used as the source of virus, and 1FAD was used as the inoculum at each passage. The results are shown in Table 7. From these and the above data it appears that P-NI (5) virus originally isolated from bronchial washings of the 5th mouse passage possesses all the properties, tested so far, of the fully adapted virus, exemplified by P-NI (15).

Table 7. *Bronchial wash passages of P-NI (5) and P-NI (15) viruses*

Virus inoculum	Test for virus HA titre of BW of passage no.			Pathogenicity					
				Lung lesion score passage no.			Percentage mortality		
	1	2	3	1	2	3	1	2	3
P-NI (5)	18	22	30	3.5	3.6	3.5	50	60	60
P-NI (15)	35	36	32	8.9	3.8	3.9	80	60	80

BW = bronchial washings

Further experiments provided direct support of the findings of Davenport (1954) that an influenza virus population has an unequal capacity for adaptation. Eight mouse lung passages of NP-I (5) and P-I (5) viruses, using bronchial washings as described above, were carried out. No change was noted in any of the *in vitro* and *in vivo* properties of these viruses as shown in Table 4. The lesion score of P-I (5) remained a value of approximately 2.8 at each mouse passage. This substrain therefore showed no capacity to mutate to the P-NI type in the above short series.

*Growth curves of NP-I (5), P-I (5), P-NI (5) and P-NI (15) viruses.* In their studies on the adaptation of influenza virus to mice, Briody & Cassel (1955) have shown that concomitantly with the ability to produce extensive pulmonary consolidation there is acquired the ability by an influenza type A prime strain to grow at an accelerated rate. In order to define further the properties characterizing the selective advantage of P-NI (15) virus, the multiplication pattern of NP-I (5), P-I (5), P-NI (5) and P-NI (15) viruses was compared.

One FAD of 42 hr. LD allantoic fluid preparations of the four viruses was inoculated intranasally into groups of mice. Four mice from each group were killed at 2 hr., 1, 2, 3 and 4 days after inoculation. Mouse lung suspensions were prepared. Egg infectivity titrations were performed with each suspension. Representative results of three experiments are given in Fig. 2. A difference greater than a 0.6 log unit between the EID<sub>50</sub> titres of two samples was taken as being significant.

The data depicted in Fig. 2 indicate that, in comparison to NP-I (5) virus, P-I (5), P-NI (5), and P-NI (15) viruses exhibit the ability to multiply to a greater extent, as well as to produce an accelerated growth curve. In contrast to P-I (5), the fully adapted virus possesses a differential reactivity with inhibitors.

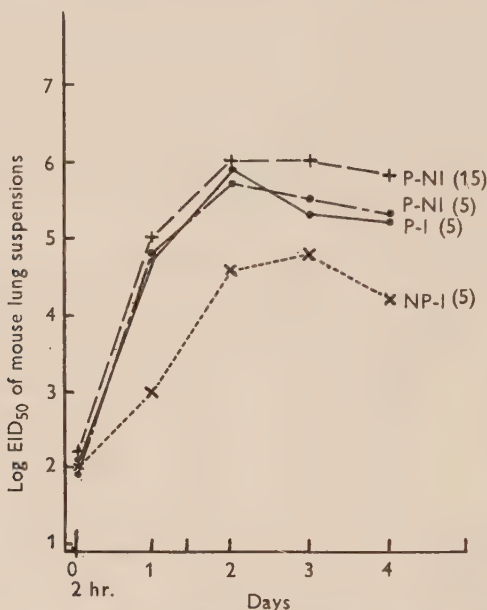


Fig. 2. Egg infectivity titrations of mouse lung suspensions at various intervals after mouse inoculation with NP-I (5), P-I (5), P-NI (5) and P-NI (15) viruses.

### DISCUSSION

The results of experiments attempting to elucidate some of the characteristics of the process of adaptation to the mouse lung of Rob virus have shown that the virus form at the end of the process differs from an unadapted form in: (a) a high and stable virulence; (b) the ability to produce an accelerated growth curve and to multiply to a greater extent; (c) the inability to be converted to the indicator phase as tested with sheep mucin and ovomucin inhibitors; (d) a marked decrease of enzymic activity on sheep mucin and mouse lung inhibitors; and (e) a shift to a high position in the fowl red cell receptor gradient.

The complexities of the interaction of active and indicator influenza viruses with mucoid macromolecules and the red cell surface have been extensively summarized by Burnet (1951*b*). The simplest hypothesis to account for the above *in vitro* properties (c, d and e) would be that adaptation to the mouse lung of Rob virus is associated with two characteristics. The preferential combination of the heated adapted virus with the red cell rather than with the inhibitor in the inhibitor sensitivity three-component test (Anderson, Burnet, Fazekas de St Groth, McCrea & Stone, 1948) indicates a change in the affinity



of adsorption to sheep mucin and ovomucin inhibitors resulting in decreased inhibitor reactivity. The inhibitor-destroying tests and altered reactivity with fowl cells treated with RDE point to a masking, or a loss, of at least some of the enzyme groups on the virus surface. There is indicated, therefore, a change in the spatial arrangement of the adsorptive and enzymically active groups on the virus surface.

As suggested from the experimental data, these adsorptive and enzymic changes may be the result of a single-step mutational change acquired by the virus particle along with the ability to produce extensive lung lesions. The question arises whether these changes represent functionally important changes in the adaptation process or whether they are 'accidentally' associated changes to those involving virulence. The growth curve studies using the different viruses isolated from the fifth and fifteenth passages may provide some evidence on this point. The results of these experiments revealed that P-I (5) virus, i.e. a virus possessing pathogenicity and indicator properties, and P-NI (15) virus, i.e. a virus which was pathogenic and a non-indicator, grew at an accelerated rate and multiplied to a greater extent than NP-I (5) virus, a non-pathogenic virus with the indicator property. Therefore, the ability of the virus to grow at an accelerated rate and to multiply to a greater extent is correlated only with the ability to produce extensive lung lesion production, and not necessarily with the adsorptive and enzymic changes noted. However, since in these experiments adaptation resulted in the selection of a virus of the type P-NI, an additional selective advantage of such a virus presumably exists. The adsorptive and enzymic changes noted above may, accordingly, be a reflexion of the mutation of a virus particle to a decreased reactivity with the inhibitor, or inhibitors, present in the lung tissue during the course of infection. Such a particle would then make a more rapid contact with the susceptible cells than a non-mutated one. This may be, in turn, correlated with a shortened lag phase in the growth cycle. Growth curve determinations at intervals of less than 24 hr. should provide evidence for this point. On the other hand, other unknown mutations of the virus particle may be important determinants of the selective survival advantage of P-NI type virus.

In the O-D transformation of influenza virus (Stone, 1951), the two phases exhibit a differential behaviour against soluble inhibitors. Multiplication in the amniotic cavity of the chick embryo gave rise to mutants which differed in their agglutination of fowl red cells, weakly at first, and in being able to multiply in the allantoic cavity. O-phase virus was enzymically active against human ovarian cyst mucin but not against ovomucin of chick embryo lung. D-phase virus acted on all three inhibitors. After transformation to the indicator state, O virus was inhibited only by the human mucin, while D virus was inhibited both by human and avian mucins. These changes of the adsorptive-enzymic properties of the O-D transformation are plausibly explained as having evolved in relation to the multiplication of the virus in the chick embryo. The changes of the adsorptive-enzymic mechanism in the present study did not follow a similar pattern. It was seen that after transformation to the indicator state both the unadapted and adapted Rob viruses were

inhibited by bronchial washings and mouse lung preparations. Furthermore, under certain experimental conditions, adapted virus showed a reduced enzymic activity against the mouse lung inhibitor, while unadapted virus was enzymically active. In elaboration of the discussion in the previous paragraph, it may be that the changes in the adsorptive-enzymic mechanism found above are related to a decreased reactivity with intracellular mucoid receptors. The possibility of intracellular receptors playing a part in infection has been raised by Edney & Isaacs (1950).

According to Burnet (1956) there is a gradual change of the virus in the chick embryo from O to D, and not a simple replacement of O virus by an increasing proportion of D virus particles. At least two intermediates have been postulated. The present findings indicate that at least two 'intermediates' are present in bronchial washings collected from an early stage of adaptation. In contrast to the fully adapted virus which could not be converted to the indicator state as tested with sheep mucin inhibitor, two viruses were isolated: (a) a virus which was non-pathogenic and could be converted to the indicator state; and (b) a virus which was pathogenic, but not to the extent shown by the fully adapted virus, and which also could be converted into an indicator.

In addition to these two viruses, a virus possessing all of the tested properties of the fully adapted virus was isolated from the bronchial washings collected from an early stage of adaptation. This provides some experimental evidence in support of previous hypotheses stating that adaptation results from the selection of mutants, whether occurring during mouse passage, or present in the original inoculum, or both. However, that other processes may also operate in the adaptation process is indicated from an inspection of the course of adaptation in Fig. 1, where only a gradual rise in pathogenicity of the virus population is evident. If adaptation consisted merely of a selection of a particular mutant, a more rapid acquisition of extensive lung pathogenicity might be expected. In line with the hypothesis of Davenport (1954), one can envisage a more dynamic state, resulting in the development of many mutant forms of virus. Further mutations of various intermediate forms might take place, and interference and competition of these for the susceptible cells would occur. Genetic interaction between various virus forms might also be expected to occur, as is indicated by the experiments of Burnet & Lind (1954). While it would appear that mutations are the primary factors in mouse lung adaptation, a Lamarckian change may also operate.

A diversity of behaviour in the study of mouse adaptation has been encountered by various investigators. Hirst (1947) has shown that the enhancement of virulence with mouse passage of an influenza A virus is not due to an increasing ability of the agent to grow in the lung. In their studies using an influenza A prime strain, Burnet & Lind (1954) have observed a variable delay in the appearance of any lesion-producing capacity and, once this capacity has appeared, a rapid increase to maximal activity occurs. Adaptation is envisaged as a steplike process with many inheritable grades. It appears probable that different mechanisms operate for different viruses in regard to virus population changes in the adaptation process.

It seems likely, however, that adaptation of influenza virus to the mouse lung may conform to the broad meaning indicated by Stanier (1953) in relation to adaptation among micro-organisms. The totality of processes enabling a heterogeneous virus population to multiply in a new environment would include both genetic and environmental factors, including the selection of a virus better fitted to adapt to the new surroundings.

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#### ADDENDUM

After this manuscript was completed, a paper was published with data relevant to the above work (Medill-Brown, M. & Briody, B. A., 1955, Mutation and selection pressure during adaptation of influenza virus to mice, *Virology*, 1, 301). The authors conclude that the process primarily taking place during mouse lung adaptation of an influenza B strain is a selection of mutants resistant to B-inhibitor.

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## Nutritional Patterns in Acetic Acid Bacteria

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**SUMMARY:** The nutritional patterns of 28 typed and untyped strains of *Acetobacter* spp. ranged between extremes with predominant lactate (lactaphilic) or glucose (glycophilic) metabolism. Typical lactaphilic strains did not require exogenous supplies of growth factors and gave rise to new ninhydrin-reacting substances when cell suspensions were incubated in solutions of proline, glutamate and aspartate. Under similar conditions, glycophilic strains yielded no new ninhydrin-reacting substances and required added nicotinate, pantothenate and, in some cases, *p*-aminobenzoate for growth.

All strains utilized ammonium sulphate as sole source of nitrogen for growth. Under such circumstances, most lactaphilic strains grew readily when lactate provided the only source of energy and carbon: other organic acids, even to be partially effective as substitutes for lactate, required the simultaneous presence of glucose. Glycophilic strains utilized ammonium sulphate as sole source of nitrogen for growth slowly and to a limited extent in glucose medium, the degree of growth being increased by the presence of certain organic acids. Characteristically, glycophilic strains required certain sugars or sugar alcohols for growth, but were little or not at all stimulated by lactate.

Working with 7 strains of acetic acid bacteria, Rainbow & Mitson (1953) distinguished organisms having either a predominant lactate metabolism (the *Acetobacter mobile* group) or a predominant glucose metabolism (the *A. viscosum* group). In this work, we prefer to refer to the former as the 'lactaphilic' and to the latter as the 'glycophilic' group. The validity of the distinction between these groups has now been extended by tests on a further 21 strains of *Acetobacter* spp. The distinction is shown to be associated, circumstantially at least, with differences in respect of requirements for exogenous growth factors and in behaviour of cell suspensions in solutions of amino acids biochemically related to L-glutamic acid.

### METHODS

*Test organisms and inocula.* The organisms used in this work were the strains of *Acetobacter* used by Rainbow & Mitson (1953) and the following: *Acetobacter aceti* (NCTC 6423), *A. acetosum* (NCTC 2224), *A. ascendens* (NCTC 4937), *A. orleanense* (NRRL B 55), *A. oxydans* (NCTC 6720), *A. rancens* (NRRL B 65), *A. suboxydans* (NCTC 6430), *A. suboxydans* (NCTC 7113) and the untyped strains D1, D2, D3, D4, D5, D6, D12, G1, A1, A2, A3, A4 and A5. The unclassified strains were isolated from beer or brewers' pitching yeasts by plating on malt agar under aerobic conditions. Each undetermined isolate produced a surface film or pellicle when grown on liquid media and consisted of catalase-positive, Gram-negative rods which oxidized ethanol to acetic acid during growth in 10% malt extract containing 2% (v/v) ethanol. Stock cultures

were maintained and inocula for growth tests prepared according to Rainbow & Mitson (1953) with the modification that certain inocula, treated similarly after harvesting, were grown in clarified 10% malt extract. For work with suspensions, organisms were grown in 6 ml. portions of medium for 2–4 days, and the entire subculture transferred to 60–100 ml. of medium I contained in a conical flask (250 ml. capacity). After incubation at 28° for 6–7 days, the organisms were harvested (centrifuge), washed twice with sterile 0.85% (w/v) NaCl solution and resuspended in 30–50 ml. of the same solution, aseptic conditions being observed throughout.

*Media.* (a) Clarified malt extract was prepared by autoclaving (15 lb./sq.in. for 7 min.) a 10% (w/v) solution of dried malt extract (Muntona Ltd., Bedford) with *c.* 0.5 g. Celite filter-aid/100 ml. of extract. This mixture was stored in the refrigerator for 3 days and then filtered through Whatman no. 42 paper.

(b) Medium I was the basal medium *a* described by Rainbow & Mitson (1953) but for tests involving glycophilic strains lactic acid was omitted and 1.0 mg. each of adenine and uracil/100 ml. were added. Media II, III and IV were identical with I, except that constituents were omitted as follows: glucose (from II), casein acid hydrolysate (CAH; from III) and glucose and CAH (from IV). In addition, the concentration of ammonium sulphate in media III and IV was increased to 0.3 g./100 ml.

*Materials.* Sugars, sugar alcohols and organic acids were of Laboratory Reagent grade excepting the following, which were of A.R. grade: citric, lactic and succinic acids, and sodium acetate (3H<sub>2</sub>O) which was used for all experiments involving acetate. The amino acids were commercial preparations, the purity of which was examined by paper chromatography. The L-proline invariably contained a trace of hydroxyproline and the DL-alanine invariably contained glycine, from which it was partially separated by recrystallization from aqueous acetone. Occasionally, samples of L-glutamic acid and L-arginine contained traces of aspartic acid and ornithine respectively. The presence of all such impurities was allowed for in interpreting the results obtained with suspensions of organisms.

The CAH used in media was the 'vitamin-free' sample (Allen and Hanburys Ltd.) reported on by Moore & Rainbow (1955).

*Growth tests* were carried out as described by Rainbow & Mitson (1953). In a few cases, when strains A1, A2, A3, A4, A5 and G1 were grown on certain substrates, the organisms formed coherent pellicles at the surface, so that only a crude visual assessment of growth was possible (e.g. in Table 1). Growth tests were allowed to proceed for 7 days, Spekker turbidity readings being taken after 48 hr. and thereafter daily. Turbidity readings reported in this paper refer to measurements made after 7 days unless otherwise stated. However, such readings sometimes conceal certain kinetic aspects of growth, particularly with respect to growth on media supplemented with organic acids. For example, certain strains (Table 1) which grew on acetate or ethanol did so only after a considerable lag and *Acetobacter mobile* grew on glucose + lactate (Table 2) only after 6 days of incubation.



The pH values of the culture fluids of all tubes in growth tests were determined colorimetrically after 7 days.

*Experiments with suspensions of organisms* were carried out at 28° and initial pH 5.8 in systems consisting of 3 ml. of substrate solution + 3 ml. of suspension. The latter was prepared as above and, when necessary, previously diluted with further sterile saline so that the final reaction mixture had an optical density value of 1.0–1.6. The substrates were solutions at pH 5.8 of 0.6% (w/v) of the L or 1.2% (w/v) of the DL forms of the following amino acids: L-proline, L-glutamic acid hydrochloride, L-aspartic acid, DL-alanine and L-arginine hydrochloride. In some experiments, the amino acid solution was supplemented with glucose and/or potassium lactate (pH 5.8) added in the form of two drops (*c.* 0.05 ml.) of sterile 50% (w/v) solutions/6 ml. test solution. Each experiment included the appropriate uninoculated blanks and a blank consisting of 3 ml. of a suspension of the test organism + 3 ml. of sterile 0.85% (w/v) saline. Samples (10  $\mu$ l.) of supernatant fluid were withdrawn after 48 hr. (and when necessary at subsequent daily intervals up to 7 days), and examined by paper chromatography for content of amino acids as described by Chamberlain & Rainbow (1954). The pH values of the culture fluids were determined colorimetrically at the end of the incubation period.

## RESULTS

### *Carbon and energy requirements*

The ability of certain sugars, sugar alcohols and organic acids to promote the growth of the test organisms was determined by growth tests in media II, III and IV. Solutions of test substances were adjusted, when necessary, to pH 5.8 with KOH solution before addition to the test medium. Tests were carried out in these media in order to obtain information concerning the utilization of the test substances as sources of (a) energy (in medium II); (b) carbon fragments (in medium III) and (c) energy and carbon fragments (in medium IV).

The results showed that each test strain fitted into one or other of the two groups distinguished by Rainbow & Mitson (1953) and are illustrated (Table 1) by data for three strains selected as representative of all the strains tested.

The *lactophilic* group comprised the following strains: *Acetobacter aceti*, *A. acetosum*, *A. ascendens*, *A. orleanense*, *A. oxydans*, *A. rancens*, *A. suboxydans* 6430, *A. suboxydans* 7113 and strains D1, D2, D3, D4, D5, D6, D12 and G1. To these should be added Rainbow & Mitson's *A. mobile* group (*A. acidum-mucosum*, *A. mobile* and *A. suboxydans* 7069), which they closely resembled. They showed the following general features.

(a) In medium II, growth was stimulated in all cases by lactate, acetate or ethanol, lactate usually being the most effective. The sugars or sugar alcohols were completely or relatively ineffective or, like glycerol, effective only for a few strains. Growth was also stimulated to a small extent by organic acids other than lactate and acetate. The acids effective in this respect differed with the strain, succinate being the most often, and citrate the least often, effective.

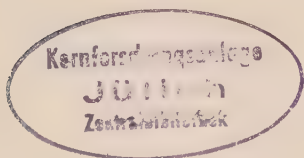
Since medium II contained potential fragments for biosynthesis (as CAH) those substances which promoted growth were likely to do so because they provided a suitable source of energy. This, however, is not necessarily so, as was shown by the fact that some growth took place in unsupplemented medium II, which was therefore itself not devoid of an energy-yielding material.

Table 1. *Sugars, sugar alcohols and organic acids as sources of carbon and energy for Acetobacter strains*

Growth tests were made at 28° in medium I less lactate and glucose (medium II), less lactate and CAH (medium III) and less lactate, glucose and CAH (medium IV). Glucose was tested at 2% concentration and other substrates equimolar to it. Growth is recorded as excess optical density value over that of the appropriate blank: 0 = < 0.20; 1 = 0.21–0.50; 2 = 0.51–0.80; 3 = > 0.80; + = growth observed to have occurred, but no quantitative measurement was possible.

Substrate	Strain of <i>Acetobacter</i>								
	D 3 (lactaphilic)			<i>A. ascendens</i> (lactaphilic)			A 1 (glycophilic)		
				Growth in medium					
	II	III	IV	II	III	IV	II	III	IV
Glucose	0		0	0	0	0	3		1
Fructose	0	0	0	0	0	0	+	0	2
Mannitol	0	0	0	0	0	0	+	0	2
Sorbitol	0	0	0	0	0	0	+	0	2
Glycerol	1	0	0	0	0	0	+	0	2
Ethanol	2	0	0	2	0	0	+	0	0
Acetate	2	0	0	1	0	0	0	0	0
Citrate	0	0	0	0	0	0	0	2	0
Fumarate	0	0	0	0	0	0	0	1	0
$\alpha$ -Ketoglutarate	0	1	0	1	0	0	0	1	0
Lactate	3	3	2	3	0	0	1	1	0
Malate	0	1	0	1	0	0	0	1	0
Succinate	1	3	0	1	0	0	0	0	0
Growth of blank (optical density value)	0.41	0.01	0.01	0.29	0.02	0.01	0.21	0.57	0.01

(b) In medium III, which contained a potential source of energy (glucose) and ammonium sulphate as sole source of nitrogen, lactate was again the best substrate for growth, except for *Acetobacter ascendens* and *A. oxydans* (see under (c) below). Other organic acids (but never acetate) also stimulated growth, succinate being the most effective, as well as being effective for the greatest number of strains (13). The other acids usually gave smaller stimulations and were effective for fewer strains as follows:  $\alpha$ -ketoglutarate (12), malate (10), citrate (4) and fumarate (2). The number, and frequently the magnitude, of the stimulatory effects promoted by organic acids, other than lactate and acetate, in medium III were greater than those promoted by the same acids in medium II. This suggests that these acids were more generally utilized as sources of carbon, rather than as sources of energy, by the lactaphilic strains. On the other hand, acetate and ethanol appeared to be sources



of energy but not of carbon. The sugars and sugar alcohols were also ineffective as sources of carbon.

(c) In medium IV, only lactate supported growth. Thus, only lactate was available as a source of both energy and carbon for the lactaphilic strains. *Acetobacter ascendens*, *A. oxydans* and strain D2 were, however, exceptional and failed to grow in medium IV, no matter what supplement was added. It appears that, to obtain growth of *A. ascendens* and *A. oxydans* in a medium containing ammonia as sole source of nitrogen, binary mixtures of carbon compounds must be supplied (see below). However, it seemed reasonable to group these strains with the lactaphiles because they utilized lactate readily in medium II, and also because they grew in the absence of added growth factors (see below).

The *glycophilic* group comprises strains A1, A2, A3, A4 and A5. In medium II, best growth was obtained on glucose, mannitol, sorbitol and glycerol: fructose was also readily utilized for growth in most cases. In contrast to that of the lactaphilic group, growth of the glycophilic group in media II and IV was little, if at all, stimulated by lactate. In the presence of glucose but absence of a major source of organic nitrogen (medium III), only A1 and A2 grew at all well, and growth was stimulated to some extent by citrate, fumarate,  $\alpha$ -ketoglutarate, lactate or malate, but not by acetate or succinate. Strains A3, A4, and A5 were scarcely or not at all stimulated by organic acids in any of the media: they grew rapidly and well only in presence of CAH. With these glycophilic strains should be grouped *Acetobacter capsulatum*, *A. gluconicum*, *A. turbidans* and *A. viscosum* which comprise Rainbow & Mitson's *A. viscosum* group and which most closely resembled A1 and A2 in their response to organic acids; in addition, *A. turbidans* and *A. viscosum* were stimulated by succinate in medium III.

*Binary mixtures as sources of energy and carbon.* Whilst lactate was a source of energy and carbon for most lactaphilic strains, there were indications, described above, that the other organic acids provided either energy or carbon, but not both, for many of the test organisms. This possibility was tested further by growth tests in medium IV, in which all possible pairs of the following substrates were presented to the test organisms: glucose, glycerol, ethanol, acetate, citrate, fumarate,  $\alpha$ -ketoglutarate, lactate, malate, succinate. For these tests *Acetobacter ascendens*, *A. oxydans*, and Rainbow & Mitson's 7 strains were used.

The results (examples in Table 2) showed that: (a) binary mixtures which promoted the growth of the lactaphilic strains *Acetobacter acidum-mucosum*, *A. mobile* and *A. suboxydans* (NCTC 7069) contained either citrate, fumarate, malate or succinate (but not  $\alpha$ -ketoglutarate) as one substrate (presumably providing carbon fragments) and either glucose, glycerol, ethanol or acetate as the other. Since *A. acidum-mucosum* and *A. suboxydans* NCTC 7069 grew well on lactate as sole source of carbon and energy, binary mixtures containing lactate also supported good growth. However, for growth of *A. mobile* on lactate in medium IV, a second substrate (glucose or glycerol) was required; growth then occurred after a considerable lag. The nature of these second



substrates implied that lactate provided a source of carbon. Lactate may also provide a source of energy for *A. mobile*: thus it grows well in medium II containing lactate (Rainbow & Mitson, 1953).

Not all pairs of the above substrates gave growth: thus, the growth of *Acetobacter acidum-mucosum* was not promoted by glucose or by glycerol, nor that of *A. mobile* by ethanol, no matter what other substrate was also present. Utilization of acetate for growth was observed only with *A. acidum-mucosum* when malate was the second substrate and, after a lag period of 6 days, with *A. suboxydans* when glucose was present.

Table 2. *Utilization of ammonia for growth of Acetobacter strains in presence of binary mixtures of organic compounds*

Growth tests were made at 28° for 7 days in medium IV. G=glucose, T=glycerol, E=ethanol, A=acetate, C=citrate, F=fumarate, K= $\alpha$ -ketoglutarate, L=lactate, M=malate, S=succinate. For other details, see legend to Table 1. Mixtures not included below failed to promote growth significantly greater than the appropriate blanks.

Strain							
<i>A. acidum-mucosum</i>		<i>A. ascendens</i>		<i>A. mobile</i>		<i>A. capsulatum</i>	
Mixture	Extent of growth	Mixture	Extent of growth	Mixture	Extent of growth	Mixture	Extent of growth
AM	2	AM	2	GM	1	GM	1
EM	3	EM	3	GS	1	GF	1
ES	3	LS	3	GL	1	GC	1
EF	3	LM	2	TM	1	GK	2
L+any		LF	2	TS	1	TL	2
other	3	LK	3	TL	2	TF	2
substrate				TF	1	TK	3

(b) Most binary mixtures which promoted the growth of *Acetobacter ascendens* and *A. oxydans* contained lactate (presumably energy-yielding) as one component and either  $\alpha$ -ketoglutarate, malate, succinate or (for *A. ascendens* only) fumarate. These strains resembled *A. acidum-mucosum* in that they grew on ethanol+malate mixtures and failed to utilize glucose for growth.

(c) Binary mixtures promoting the growth of the glycophilic strains (*Acetobacter capsulatum*, *A. gluconicum*, *A. turbidans* and *A. viscosum*) always contained glucose or glycerol as one, presumably energy-yielding substrate and, except for *A. capsulatum*, glucose+glycerol also stimulated growth to a small extent. The other substrates complementary to glucose or glycerol were organic acids (never acetate) of which  $\alpha$ -ketoglutarate was particularly effective.

*Variations of pH value during growth.* Since media II, III and IV were not well buffered over the range pH 4–6, the possibility was considered that the smaller beneficial effects of organic acids on growth might be due to buffering action, which would be particularly effective in medium III against the acid end products (e.g. gluconic acid) formed from glucose. Whilst organic acid cations, as buffers, may assist growth, the stimulations produced by them cannot be ascribed only to that cause. For example, in medium III, citrate, fumarate or acetate exerted appreciable buffering action but failed to stimu-

late the growth of D3. The same is true for acetate and succinate with A1. Similar examples could be selected from the other test organisms, and it is concluded that the stimulation of growth produced by organic acids was due primarily to their participation in metabolism.

The pH values also showed that (a) the metabolism of organic acids in medium II, and of lactate in medium IV, by lactaphilic strains produced more alkaline products; (b) glucose was metabolized to acid end products by all strains, in many cases even when appreciable growth had not occurred (e.g. D3 in medium IV).

#### Nitrogen requirements

The tests designed to determine the carbon and energy requirements also give information concerning the nitrogen requirements of test organisms. All strains grew well on CAH and the ability to utilize ammonia as sole source of nitrogen has been discussed above (media III and IV).

Table 3. *Amino acids as source of nitrogen for the growth of Acetobacter strains*

Growth tests were made in defined glucose + lactate + salts medium (series A) or glucose + 0.2%  $(\text{NH}_4)_2\text{SO}_4$  + salts medium (series B). Growth is recorded as optical density values  $\times 100$  after 7 days of incubation at 28°. Concentrations (g./100 ml.) of substrates were: series A, amino acids, 0.6;  $(\text{NH}_4)_2\text{SO}_4$ , 0.3; series B, DL-alanine, 0.72; other amino acids and CAH, 1.2.

Strain	Growth on						
	No added nitrogen	L-Glutamic acid	L-Aspartic acid	DL-Alanine	L-Proline	$(\text{NH}_4)_2\text{SO}_4$	CAH
Series A							
<i>A. acidum-mucosum</i>	1	84	102	114	150	82	—
<i>A. mobile</i>	1	120	92	21	152	28	—
<i>A. suboxydans</i> 7069	1	145	155	126	170	130	—
<i>A. ascendens</i>	2	118	123	3	165	4	—
<i>A. oxydans</i>	2	116	95	5	154	3	—
Series B							
<i>A. capsulatum</i>	.	119	63	5	81	22	130
<i>A. gluconicum</i>	.	73	41	7	39	17	92
<i>A. turbidans</i>	.	57	39	20	58	26	84
<i>A. viscosum</i>	.	40	51	16	40	22	72

*Single amino acids as sources of nitrogen.* Preliminary studies by paper chromatography indicated that glutamate, proline, aspartate and  $\alpha$ -alanine were the amino acids most rapidly assimilated by acetic acid bacteria, particularly by lactaphilic strains, during growth on media containing CAH. Further tests on five lactaphilic strains grown on medium I, the CAH and ammonium sulphate of which was replaced by single amino acids, showed that good growth was obtained on L-proline, L-glutamate or L-aspartate, and sometimes on DL-alanine. The other 14 amino acids tested supported little or no growth, with few exceptions (e.g. L-arginine for *Acetobacter mobile* and L-leucine for *A. oxydans*). Similar tests on glycophilic strains, but in which 0.2% (w/v)  $(\text{NH}_4)_2\text{SO}_4$  was included in the basal medium, showed that proline,

glutamate and aspartate permitted amounts of growth of the order of those produced by CAH (Table 3). These tests indicated that L-glutamic acid and the amino acids biochemically related to it were key nitrogen compounds in the nutrition of acetic acid bacteria.

#### *Incubation of suspensions of organisms with amino acids*

When thick, non-proliferating suspensions of all test strains were incubated with solutions of proline, glutamate and aspartate, and the super-natant fluids were examined by paper chromatography, a clear difference between lactaphilic and glycophilic strains was observed as follows.

(a) No new ninhydrin-reacting material appeared in suspensions of glycophilic strains either in the absence of added supplements or in the presence of lactate, glucose, glucose + lactate or, in one case tested (*Acetobacter capsulatum*) in the presence of the full complement of growth factors as included in medium I.

(b) With all lactaphilic strains, new ninhydrin-reacting material, not detected in the control suspensions, appeared. The chief of these new materials in the proline, glutamate and aspartate systems possessed the chromatographic mobilities respectively of glutamate, aspartate and alanine.

Neither lactaphilic nor glycophilic strains yielded new ninhydrin-reacting materials when suspended in solutions of  $\alpha$ -alanine.

#### *Requirements for added growth factors*

The growth factor requirements of the strains hitherto unexamined in this respect were determined as described by Rainbow & Mitson (1953). The results showed that all strains already classified as lactaphilic had no requirement for added growth factors. By contrast, all glycophilic strains required added pantothenate and nicotinate; strain A3 also required *p*-aminobenzoate for growth. The pattern of growth factor requirements found by Rainbow & Mitson (1953) was therefore confirmed, and its relevance extended, to a further 21 strains of *Acetobacter* spp. The requirement of *A. gluconicum* for an additional unidentified growth factor (noted in the earlier work) was satisfied by uracil, which was not replaceable by cytosine, thymine or orotic acid. In the absence of uracil, *A. gluconicum* grew only after a lag of nearly 7 days, but organisms from such cultures, on transfer to uracil-free medium I, grew almost as well as in the same medium containing uracil, suggesting that selection for uracil-independence had occurred.

*Note on p-aminobenzoate requirements.* Neither pteroylglutamic acid nor folinic acid SF replaced *p*-aminobenzoate for the growth of *Acetobacter capsulatum*, *A. gluconicum*, *A. turbidans* and *A. viscosum*. However, the concentration of *p*-aminobenzoate needed to produce half-maximum growth of these strains was decreased by adding purines (of which hypoxanthine was the most effective) and, except with *A. gluconicum*, by vitamin B<sub>12</sub>. The *p*-aminobenzoate-sparing action of vitamin B<sub>12</sub> was particularly great with *A. capsulatum*, the concentration of *p*-aminobenzoate needed for half maximum growth being decreased from 13 to 5  $\mu\text{g./ml.}$  by the presence of 0.015  $\mu\text{g. B}_{12}/\text{ml.}$



## DISCUSSION

The present work provides further evidence that acetic acid bacteria may be divided into the two groups suggested by Rainbow & Mitson (1953). The essential points of difference between the groups consist in the ability of lactaphilic, but not of glycophilic, strains: (a) to oxidize lactate and acetate; (b) to yield new ninhydrin-reacting substances when cell suspensions are incubated with proline, glutamate and aspartate; (c) to grow in media devoid of added pantothenate and nicotinate.

Our division of *Acetobacter* strains may be compared with that of Vaughn (1942). Vaughn's group 1 (oxidize acetic acid) and group 2 (do not oxidize acetic acid) correspond respectively to our lactaphilic and glycophilic groups. However, Vaughn's subdivision on the criterion of utilization of ammonia as the source of nitrogen seems less satisfactory since all our strains did so when other requirements for growth were also supplied. As a point of detail, it must also be noted that Vaughn's *Acetobacter suboxydans* should fit into our glycophilic group, whereas the three (typed) strains bearing this name which were examined by us were certainly lactaphilic.

Leifson (1954) suggested the recognition of two genera of acetic acid bacteria, retaining the generic name *Acetobacter* (type species *A. aceti*) for Vaughn's group 1. These are non-flagellated or peritrichously flagellated and oxidize acetate and/or lactate. For species which do not oxidize lactate or acetate (Vaughn's group 2) a new genus *Acetomonas* (type species *Acetomonas suboxydans*) was suggested by Leifson, the members of which are either non-flagellated or have polar multitrichous flagella. Again, Leifson's genera correspond closely to our groups, his proposed genus *Acetomonas* corresponding to our glycophilic strains. Thus, our work provides nutritional and biochemical evidence in support of the groups of acetic acid bacteria observed by Vaughn and by Leifson. However, whether the several criteria, which differentiate these groups, are of sufficient evolutionary importance to merit the separation of a genus *Acetomonas* from that of *Acetobacter*, is still an open question.

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## Cultural and Biochemical Characteristics of the Genus *Chromobacterium*

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**SUMMARY:** The cultural and biochemical characteristics of 38 strains of *Chromobacterium* are described. They are non-sporing aerobic Gram-negative rods which produce a violet pigment and possess both polar monotrichous and peritrichous flagella. They produce little acidity from carbohydrates and utilize citrate.

Two main groups of strains were distinguishable, a mesophilic and a psychrophilic group. The former usually ferment glucose anaerobically, are markedly proteolytic, facultatively anaerobic, and give a positive egg-yolk reaction, but do not hydrolyse aesculin. The psychrophilic organisms produce acid from glucose but only aerobically, are poorly proteolytic, strictly aerobic, and hydrolyse aesculin but do not produce an egg-yolk reaction.

*Chromobacterium viscosum* Grimes, *C. iodinum* Davis and *C. ianthinum* Gilman have been re-examined; they should be excluded from the genus. The nomenclature of the genus is confused. Proposals have therefore been made to the International Committee on Bacteriological Nomenclature that the mesophilic species should be called *Chromobacterium violaceum* and the psychrophilic species *C. lividum*, and neotype strains have been suggested.

The genus *Chromobacterium* comprises a group of bacilli producing on ordinary media a characteristic violet pigment which is soluble in ethanol but not in water or chloroform. The genus was defined in these terms by Buchanan (1918): this is a narrow definition of the genus, excluding the *Bacillus prodigiosus* group (*Serratia*) which is not considered here. These organisms are generally known as *Chromobacterium violaceum* (in the older literature *Bacillus violaceus* or *Bacterium violaceum*). They are a poorly known group, and the classification and taxonomy are in confusion. About 70 blue or violet chromogens are described in the literature, but authentic strains of the great majority of them are not available. The work reported here is an examination of available strains which can reasonably be placed in this genus, or which have been described under this generic name. The most extensive work on the genus has been that of Bampton (1913), Calderini (1925) and Cruess-Callaghan & Gorman (1933). Early in the present work it was noted that the difference in growth temperature, emphasized by the last-named authors, divided the strains into two groups, those which grow at 37° but not at 4° and those which grow at 4° but not at 37°. This division has proved to be fairly sharp, and is correlated with a number of other characteristics. For brevity therefore they are referred to as the mesophils and the psychrophils respectively.

## METHODS

Variable results are obtained with different techniques and growth temperatures and times, and for this reason the techniques used here are described in considerable detail. All tests, except where otherwise stated, were carried out at 25°, at which temperature the growth rates of the mesophils and the psychrophils are about equal. The inability of single organisms to multiply on nutrient agar, the rapid dying-out of strains in culture and the inability of some strains to grow at high temperatures upon over-dried plates, necessitated setting up many controls, and preferably inoculation into condensation water on slopes of solid media. Many out-dated tests were performed as an aid to the revision of the nomenclature of the genus.

The source of the strains studied is shown in Table 1. Besides the 38 strains of *Chromobacterium*, three bacteria, which have in the past been placed in this genus, *Chromobacterium viscosum* Grimes 1927, *C. iodinum* Davis 1939, and *C. ianthinum* Gilman 1953, were studied since they are believed to be authentic strains. They are referred to here under the names *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI respectively (see Table 1) to avoid confusion with the true *Chromobacterium* strains, without implying that these are their correct names. Another organism which has been placed in this genus is *Chromobacterium prodigiosum* Topley & Wilson 1929 (*Serratia marcescens*), here referred to as 'Bacillus prodigiosus'; two strains (NCTC 1377 and NCTC 9493) were studied together with two strains of *Pseudomonas aeruginosa* (NCTC 2000 and NCTC 6749) for comparison. In my opinion neither organism should be classified in the same genus as *Chromobacterium*, and the results with them are only given where they are of interest. Another strain of *C. iodinum* (strain RE) which is the authentic culture of Davis, was obtained late in the investigation; it was not fully investigated, but is clearly the same organism as ATCC 9897. Strain SH is that of Black & Shahan (1938); strains SL and RV are those of Sippel, Medina & Atwood (1954); strains BH and BN are from cases 11 and 12, strains FH, MK and LG are the Malayan water strains and strain TV is the Trinidad strain of Sneath, Whelan, Singh & Edwards (1953); strain EC is strain 16 of Cruess-Callaghan & Gorman (1933); strain DK is that of Darrasse, Mazaud, Giudicelli & Camain (1955). Strains AM and TI are believed to be the strains called *C. amethystinum* and *C. ianthinum* by Gilman (1953).

*Staining and morphology*

Strains were grown upon nutrient agar at 25° and examined after 18 hr. and 4 days. For general morphology and measurements of size by an eye-piece micrometer, heat-fixed films were stained with Löffler's alkaline methylene blue. Heat-fixed films were stained by the methods of Gram, Ziehl-Neelsen, Albert and Hiss according to the details given in Mackie & McCartney (1948, pp. 82, 86, 88, 90, 94). For Gram staining, both the ethanol method of decolorizing followed by neutral red as counterstain, and the acetone method followed by safranin, were used. Fat staining was by the technique of Burdon (1946)



using Sudan Black B (G. T. Gurr Ltd., London, S.W. 6). Neisser staining was as follows: 0.1 g. methylene blue was dissolved in 2 ml. ethanol, and 95 ml. distilled water and 5 ml. glacial acetic acid were added. Heat-fixed films were stained in this solution for 2 min., rinsed with tap water and counterstained for 1 min. with 0.2% aqueous Bismarck brown. The flagella stain of Leifson (1951) was used.

Table 1. *Source of strains*

Strain letters	NCTC no.	From whom received and date	Name or no. as received	Details of isolation where known			
				Investigator	Source	Country	Date
FH	9373	Whelan, 1952	Frazer's Hill	Whelan	Water	Malaya	1952
MK	9757	Whelan, 1952	Mentekab	Whelan	Water	Malaya	1952
LG	9374	Whelan, 1952	Lake Gardens	Whelan	Water	Malaya	1952
BH	9696	—	—	Sneath	Human urine	Malaya	1951
BN	9694	—	—	Sneath	Human fatal case	Malaya	1952
AM	9370	BSC, 1952	<i>C. membranaceum amethystinum</i>	—	From NCTC in 1935	—	—
MW	9371	MWB, 1952	—	Prof. Thomas	Water	England	—
NT	7917	NCTC, 1952	NCTC 7917	Collins, FBA, strain 2	Lake water	England	1949
TV	—	HCC, 1952	<i>C. violaceum</i>	HCC	Forest soil	Trinidad	1950
LW	8683	ATCC, 1952	ATCC 7461	—	—	—	—
SH	8684	ATCC, 1952	Lewitus strain ATCC 6357	Shahan	Human infection	Florida, U.S.A.	1937
RT	8685	ATCC, 1952	Shahan strain ATCC 553	L. F. Rettger, Yale University	—	—	—
SL	9695	Sippel, 1954	Sealy	Sippel	Swine infection	Georgia, U.S.A.	1953
RV	9372	Sippel, 1954	Reeves	Sippel	Bovine infection	Georgia, U.S.A.	1952
DK	9376	Darrasse, 1955	135	Darrasse	Human infection	Dakar, Africa	1954
MH	9377	U.M., 1952	—	—	—	—	—
EA	—	Eltinge, 1955	60	Eltinge	Soil	New Hampshire, U.S.A.	1953
EB	—	Eltinge, 1955	30	Eltinge	Dust	Massachusetts, U.S.A.	1952
EC	—	Eltinge, 1955	47 (ATCC 6915)	Prof. Kluyver, strain L91	—	—	—
RU	—	—	—	Sneath	Soil	England	1955
GA	—	—	—	Sneath	Soil	England	1955
DA	—	—	—	Sneath	Soil	England	1955
NC	—	—	—	Sneath	Soil	England	1955
TA	—	HCC, 1955	<i>C. violaceum</i>	Morris, HCC	Water	Trinidad	1955
TB	—	HCC, 1955	<i>C. ianthinum</i>	—	(From Institut Pasteur, Paris)	—	—
MA	—	MWB, 1955	Ch 25	MWB	Water (sand filter)	England	1955
MB	—	MWB, 1955	Ch 27	MWB	Water (sand filter)	England	1955
MC	—	MWB, 1955	Ch 28	MWB	Spring water	England	1955
PT	—	Sippel, 1955	Porter	Sippe	Swine infection	Florida, U.S.A.	1955
HA	—	Hans, 1955	H-4	Hans	Soil	Michigan, U.S.A.	1952
HB	9796	Hans, 1955	H-24	Hans	Soil	Michigan, U.S.A.	1952
HC	—	Hans, 1955	6-15	Hans	Soil	Michigan, U.S.A.	1952
HD	—	Hans, 1955	6-20	Hans	Soil	Michigan, U.S.A.	1952
HE	—	Hans, 1955	Traunstein	Hans	Soil	Germany	1955
HF	—	Hans, 1955	Ulm	Hans	Soil	Germany	1955
IN	—	Hans, 1955	Indiana X	Indiana University	Soil	—	—
CA	—	Hans, 1955	Corpe	Corpe	Soil	U.S.A.	—
PB	—	Hans, 1955	Institut Pasteur Paris, 52227	—	Water	—	1952
GR	2416	NCTC, 1955	NCTC 2416 <i>Corynebacterium viscosum</i>	Grimes	Butter	Ireland	1927
TE	9742	ATCC, 1955	ATCC 9897 <i>Pseudomonas iodina</i>	Davis	Milk	England	1938
RE	—	NIRD, 1955	<i>Chromobacterium iodinum</i> Davis	Davis	Milk	England	1938
TI	—	HCC, 1952	<i>C. ianthinum</i>	HCC	Forest soil	Trinidad	1950

Abbreviations. The addresses of workers and of the institutes (indicated above by abbreviations) that provided strains are given in the acknowledgements. Strains RU, GA, DA and NC were isolated by the method of Corpe (1951).

To exclude the formation of heat-resistant spores, 5 ml. nutrient broth cultures and nutrient agar slopes, incubated for 7 days at 25°, were heated at 56° for 30 min. and after adding 5 ml. of fresh broth and incubating at 25° for 4 days, were plated for sterility.

#### *Cultural methods*

The peptone used throughout was Bacteriological Peptone from Evans Medical Supplies Ltd., Liverpool. Nutrient broth was prepared according to the method of Hartley (1922): the nutrient agar was this broth solidified with 1.5% (w/v) Japanese agar. The potato slopes were plugs of potato resting on wet cotton wool, steamed on 3 successive days. Gelatin stabs and gelatin plates consisted of 15 g. Bacto-Gelatin (Difco) dissolved in 100 ml. nutrient broth at 80°, cleared by holding at 80° after adding 5 ml. white of egg and filtering. The pH value was adjusted to 7.2 and the medium sterilized by steaming on 3 successive days. The stabs were inoculated from broth cultures with a needle, and both pour plates and surface-streaked plates were made; they were incubated at 20° for 14 days.

Löffler's inspissated serum slopes were prepared with sterile horse serum according to the techniques of Mackie & McCartney (1948, p. 163), and inspissated at 85° for 2 hr.; they were streak inoculated and incubated at 25° for 14 days. The blood agar used was nutrient agar with 5% (v/v) sterile defibrinated horse blood added.

#### *Resistance*

*Heat resistance.* Two methods were used. (i) 5 ml. nutrient broth cultures grown for 2 days at 25° were well shaken, and then heated in a water bath at  $56^{\circ} \pm 0.2^{\circ}$ , and a loopful removed to blood agar plates after 5, 10, 15 and 30 min. To the broth which had been heated for 30 min. was added 10 ml. fresh broth, and after incubation for 4 days at 25°, it was plated out for sterility. Plates were incubated for 4 days at 25°.

(ii) Bampton (1913) used heat-resistance to classify his strains. His method was followed as closely as possible: five loopfuls of a 3-day culture grown on nutrient agar at 20° were emulsified in 50 ml. 0.85% (w/v) NaCl, and 5 ml. of this suspension were heated for 30 min. in a water bath, either at  $42.5^{\circ}$  or at  $45^{\circ} \pm 0.2^{\circ}$ . One millilitre was then added to pour plates of nutrient gelatin, which were incubated at 20° for 4 days and the colonies counted.

*Phenol resistance.* A sample (0.8 ml.) of a nutrient broth culture grown at 25° for 2 days and then well shaken was mixed with 0.2 ml. of 5% (w/v) aqueous phenol. It was incubated at 20° and after 5 and 10 min., 0.05 ml. samples were transferred to 5 ml. lots of nutrient broth, of which a loopful was plated on to blood agar. The plates and broths were incubated at 25° for 4 days, the broths being then plated for sterility.

*Resistance to penicillin.* Nutrient agar plates containing 100 units sodium benzylpenicillin/ml. (Glaxo Laboratories Ltd., Greenford, Middlesex) were inoculated by streaking one loopful of a 2-day broth culture grown at 25°; the plates were incubated at 25° for 2 days. The mesophils were also tested on similar plates incubated at 35° for 24 hr.

*Metabolism and nutrition*

*Anaerobic growth.* Nutrient agar plates were inoculated with a loopful of 48 hr. broth culture and incubated for 4 days at 25° in a McIntosh and Fildes's jar containing hydrogen.

*Growth temperature.* Since some strains, e.g. TV, will not grow on dry agar media near the upper limit of their temperature range, all tests were made by running a few drops of a 2-day broth culture down a nutrient agar slope. Slopes were incubated at 2, 10, 16, 20, 25, 30, 35 and 37° for 1 week, and those strains growing at 37° also tested at 44° for 4 days. The fluid as well as the agar was watched for growth.

*pH value for growth.* Tests were done upon nutrient agar adjusted to pH values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and  $10.0 \pm 0.1$ , by streaking a loopful of 24 hr. broth culture on to it and incubating for 4 days at 25°. Some tests were also done in nutrient broth adjusted to various pH values from 5.0 to 10.0 in steps of 0.2, similarly inoculated and incubated.

*Utilization of citrate as sole source of carbon.* The medium of Koser (1923) and that of Simmons (1926) were used. Koser's citrate was made from analytical grade reagents in doubly glass-distilled water in chromic + sulphuric-acid-cleaned glassware and inoculated by straight wire from a young broth culture. To Simmons's medium was added 0.01 % of yeast extract (Difco), and the slopes streaked with a loopful of broth culture. Both media were incubated at 25° for 7 days, and those tubes of Koser's medium which showed growth were subcultured with a straight wire into a second tube and similarly incubated.

*Tolerance of sodium chloride.* Nutrient agar and nutrient broth containing a total of 3 and 6.5 % (w/v) of sodium chloride were inoculated with a loopful of young broth culture and incubated at 25° for 7 days.

*The catalase effect.* Nutrient agar ditch plates were prepared, the ditch containing 1 µg. pure horse liver catalase/ml. Drops of tenfold dilutions (from 1/10 to 1/10<sup>6</sup>) of 24 hr. broth cultures were run across the plate and ditch, and the plates incubated for 2 days at 25°. Organisms showing the catalase effect give, with the more dilute inocula, colonies only upon the ditch.

*Production of hydrogen cyanide.* Nutrient or blood agar plates, heavily inoculated to give growth over most of the plate and incubated at 25° for 2 days, were tested by placing the end of an indicator paper inside the plate (but not touching the medium) and replacing the lid. The indicator paper was made as follows: benzdine acetate was dissolved in boiling water and the saturated solution cooled and filtered. To the filtrate was added one-tenth of its volume of 3 % cupric acetate solution. The tip of a strip of filter paper was dipped in the mixture, and this becomes blue in the presence of HCN. Other gases giving a positive test are chlorine, bromine and hydrogen chloride, while sulphur dioxide and hydrogen sulphide inhibit the reaction (Anonymous, 1938). The paper may turn brown after 10 min., apparently due to ammonia from the cultures.

Chemical proof that the gas is hydrogen cyanide was obtained as follows: a nutrient agar slope in a large flat bottle was inoculated with 10 ml. broth



culture, and incubated at the optimum temperature for 1 or 2 days, the neck of the bottle being closed by a bung with two delivery tubes which were clamped off. After incubation a gentle stream of air was blown into the bottle and the outcoming air bubbled through 1 ml. 10% (w/v) NaOH mixed with 0.1 ml. 1% (w/v)  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ . After 10 min. this solution was heated to boiling for a few seconds, and a drop of 1% (w/v)  $\text{FeCl}_3$  added. Upon cautious acidification with 5M-HCl the colour of Prussian blue proved the presence of HCN in the culture.

*Nature of attack upon glucose.* Strains MK, LW and HB were grown in the glucose ammonium medium of Mandelstam (1954) with the lactate omitted, the first strain at 35° semi-anaerobically (in deep tube culture), the latter two at 25° aerobically (in shallow layers of medium) for 3 days. The pH values of all three cultures fell to about 5.2. After removing organisms by centrifugation, the supernatant fluid was divided into two parts. One part was evaporated to small volume and continuously extracted with ether for some hours; the ether extract was chromatographed on paper in butanol + acetic acid and in butanol + ammonia (sprayed with bromocresol green and bromthymol blue respectively). The other part was steam distilled after adding an equal volume of 2N-sulphuric acid, and the volatile acids in the distillate identified by gas-phase chromatography (James & Martin, 1952).

*Pigment.* Violacein was extracted with ethanol from nutrient agar cultures grown for a week at 25°. The pigmentation of *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI was also studied on 1% peptone water or peptone agar containing 1% glucose, sucrose or mannitol, incubated at 25°. Absorption curves were measured in a Unicam spectrophotometer, and are shown in Figs. 1 and 3 with the optical density plotted logarithmically, so that curves of different concentrations of the same substance are parallel.

*Growth requirements.* Some strains were tested by inoculating with a needle from young broth cultures into the medium of Mandelstam (1954) containing lactate but without glucose, with precautions against contamination with growth factors. When growth occurred the tubes were subcultured into a second tube of medium with a needle. Cultures were inoculated at 30° for mesophils and 20° for psychrophils.

#### *Fermentation of carbohydrates*

Fermentation reactions were studied in 1% peptone water containing 0.5% of sodium chloride, 1% of carbohydrate and 1% of Andrade's indicator (Mackie & McCartney, 1948, p. 159). Durham tubes were included to detect gas. These media were sterilized by steaming on 3 successive days. The nature of the attack on carbohydrates was studied in the medium of Hugh & Leifson (1953) with glucose, mannose and fructose. All tubes were incubated at 25° for 14 days before discarding.

#### *Biochemical tests*

*Litmus milk.* Prepared according to Mackie & McCartney (1948, p. 163). Cultures were incubated at 25° for 2 weeks.

*Indole production.* Peptone water cultures incubated for 5 days at 25° were shaken with ether and tested with Böhme reagents as given in *Topley & Wilson's Principles of Bacteriology* (1946, p. 367).

*Ammonia, methyl red and Voges-Proskauer tests.* These were done by the techniques given in *Topley & Wilson's Principles of Bacteriology* (1946, pp. 368-9) after 5 days of incubation at 25°.

*H<sub>2</sub>S production.* Lead acetate paper was placed between the plug and the side of the tube of a nutrient broth culture and observed for 7 days at 25°.

*Methylene blue reduction.* Tested on a 5 ml. nutrient broth culture grown at 25° for 24 hr., by adding one drop of 1 % aqueous methylene blue and incubating for 1 hr. at 25°.

*Reduction of nitrate to nitrite.* Duplicate cultures in nutrient broth containing 0.1 % KNO<sub>3</sub> and 0.25 % agar were incubated at 25° and tested after 1 and 5 days by addition of Griess-Ilosvay reagents (using dimethyl  $\alpha$ -naphthylamine instead of  $\alpha$ -naphthylamine) as described in *Topley & Wilson's Principles of Bacteriology* (1946, p. 368). All negative reactions were checked by adding zinc dust to determine whether any nitrate remained (ZoBell, 1932).

*Destruction of nitrite.* Duplicate cultures in nutrient broth containing 0.0005 % NaNO<sub>2</sub> and 0.25 % agar were incubated at 25° and tested after 1 and 5 days in the same way as the preceding test. Absence of a pink colour showed disappearance of the nitrite.

*Catalase.* A loopful of growth on nutrient agar (grown for 2 days at 25°) was stirred into a drop of hydrogen peroxide solution (20 vol. %) and observed for bubbles.

*Urease.* Urea agar slopes (Christensen, 1946) were incubated at 25° for 4 days.

*Phosphatase.* The method of Bray & King (1943) was used and readings made after 2 days at 25°.

*Hydrolysis of aesculin.* Iron was used as the indicator of hydrolysis. The medium consisted of 1 % peptone, 0.1 % aesculin and 0.05 % ferric citrate (scales) in distilled water. Both this liquid medium in tubes, and plates of the medium solidified with 1.5 % of agar, were inoculated and observed at 25°, the tubes for 14 days and the plates for 4 days. The media were sterilized by autoclaving at 115° for 15 min.

*Haemolysis.* Growth on horse blood agar plates was examined for zones of haemolysis after incubation at 25°.

*Hydrolysis of caseine, gelatin and starch.* These were tested on cultures grown for 2 days at 25° upon skim-milk plates, and for 4 days on gelatin plates and starch plates by the methods of Smith, Gordon & Clark (1952, pp. 41, 43).

*Egg-yolk reaction.* Tested upon egg-yolk plates (Knight & Proom, 1950), consisting of nine parts of nutrient agar and one part of a Seitz-filtered mixture of one egg yolk in 250 ml. physiological saline. Plates were inoculated and incubated at 25° for 4 days.

*Utilization of malonate and production of phenylpyruvic acid.* Tested in the combined medium of Shaw & Clarke (1955) after growth at 25° for 2 days.

*Gluconate test.* Tested in the modified Hayne's medium of Shaw & Clarke (1955) in shallow layers (3 ml. in tubes of 22 mm. internal diameter). After growth for 4 days at 25° unshaken, 1 ml. of Benedict's Qualitative Reagent for glucose was added, and after standing for 10 min. at room temperature, the tubes were heated at 100° for 10 min.

*Arylsulphatase.* Tested in peptone water containing 0.001M-potassium phenolphthalein disulphate (Whitehead, Morrison & Young, 1952) after growth for 7 days at 25°, by adding M-NaOH drop by drop.

## RESULTS

### *Staining and morphology*

*Morphology.* All the strains were rods with rounded ends. At 18 hr. of incubation the rods of the majority of the mesophils were small, often coccobacillary, averaging about  $0.75 \times 2 \mu$ . in dimensions, while the rods of most of the psychrophils were larger, averaging  $1.0 \times 3.7 \mu$ . However, strain AM had large rods and strain MB small ones. After 4 days growth the difference was less marked, the sizes in the two groups averaging  $0.75 \times 2.1$  and  $0.85 \times 2.5 \mu$ . respectively. In most strains the organisms were single or in pairs, but a few, mainly psychrophils, showed chains of five to ten members. There was little pleomorphism but the longer organisms were frequently slightly curved.

*Motility.* All the strains of *Chromobacterium* examined were motile in broth cultures. The flagellation showed characteristic features: there was a single polar flagellum, and in addition one or more long subpolar or lateral flagella, which generally stained more readily and had a shorter wavelength than the short polar flagella. The lateral flagella were most abundant in young agar cultures, and were scanty in broth cultures, and they differed antigenically from the polar flagella (Sneath, 1956a). In young nutrient agar plate cultures (18 hr. at 25°) the peritrichous flagella were observed in all strains except FH, AM, MW, MH, MC and HF. The only strains in which it was difficult to demonstrate polar flagella were NT and PT. These differences in flagellation do not correlate with other features of the strains, and are not useful in dividing the group.

*Staining.* The mesophils generally showed bipolar staining with Löffler's methylene blue, with an oval pale central area; the psychrophils commonly showed barred staining, also with some oval pale areas. The difference may depend on the length of the rods. Four-day cultures showed occasional bizarre forms, but the bipolar and barred staining persisted.

All the strains of *Chromobacterium* examined were Gram-negative at 18 hr. and 4 days, except for HA, EC, MA and MW, which tended to be Gram-variable in young cultures. There were commonly Gram-positive granules (discussed below). None of the strains was acid-fast. A few acid-fast spore-like bodies were seen in strains EC, HB, CA, NT, GA and AM, but they were probably not spores. Many strains, chiefly the gelatinous ones, showed evidence of intercellular slime, but no clearly defined capsules were observed by Hiss staining. Fat globules were present in most organisms after 18 hr., being



larger and more abundant in the mesophils (except for strains AM, LW, SH and RT) than in the psychrophils. Strain HE, however, showed abundant fat. After 4 days both groups showed moderate amounts of fat. The globules were mainly subpolar, not central like the vacuoles. 'Metachromatic granules' were commonly seen at the poles, but their metachromasia was doubtful, since blue granules were not often observed in Neisser stained films, although brown granules occurred commonly. The only strain which showed unmistakable blue granules was EC, in which they were large and abundant. The polar granules may have been the structures which retained the Gram stain (often for several minutes decolorization with ethanol) and which were visible in more than half the strains of both mesophils and psychrophils; they were present in old as well as young cultures. Similar granules were seen in *Pseudomonas aeruginosa* NCTC 2000.

*Spores.* Reports of spores are found in many of the old descriptions, but none has been confirmed as heat-resistant. In my experience the spore-like bodies are not resistant to heating at 56° for 30 min., nor do broth or agar cultures survive this treatment.

*Other strains.* *Corynebacterium viscosum* and *Pseudomonas iodina* are very similar to one another. Both were rods (measuring about  $0.8 \times 2 \mu$ .) showing pleomorphism, with clubbed ends and arrangement in irregular or palisade-like clumps. Both were Gram-positive (including strain RE) in young cultures though they were Gram-variable after 4 days. They showed barred staining, were not acid-fast, were non-motile and non-sporing. *Corynebacterium viscosum* showed some visible fat, a definite capsule and well-marked metachromatic granules; *Pseudomonas iodina* showed none of these. Strain TI was polar flagellated (lophotrichous), Gram-negative and not acid-fast. It measured  $0.9 \times 2.5 \mu$ . after 18 hr., showed bipolar staining, moderate fat but no metachromatic granules, spores or capsules.

#### Cultural behaviour

*Nutrient agar plates.* The colonial appearances are very variable, since colony form depends in large measure upon the 'roughness' of the strain. The most smooth mesophils, which showed little auto-agglutination in saline suspensions and gave turbid growth in broth, produced round, low convex colonies with entire edge and a smooth shiny surface. The more rough mesophils (strains AM, MW, LW, SH, RT and RV) which auto-agglutinated readily and gave little turbidity in broth, produced raised or low conical colonies with a polygonal outline and undulate or erose edge; the surface was copper-beaten or rough, and dull and granular.

None of the psychrophils studied appeared very rough, and they gave round convex colonies, occasionally umbonate, with entire edge and smooth shiny or matt surface. A few strains threw off rougher variant colonies.

The structure of the colonies of the *Chromobacterium* strains examined was amorphous with finely granular centre, and they were semi-transparent. Colonies of gelatinous strains, only seen among psychrophils, were viscid and

jelly-like, and after 4 or 5 days they could be detached whole with a needle; they were difficult to emulsify. The non-gelatinous strains were butyrous and easily emulsified.

As a rule the colonies were 1–1.5 mm. in diameter after 2 days at 25°. Most of the mesophils produced colonies of this size in 24 hr. at 37°. Pigmentation started in the centre of colonies of the mesophils, was slight in 24 hr. at 37° and marked in 48 hr. At 25° pigmentation in both groups was delayed until the second or third day. Some psychrophils showed pigmentation first at the edge of the colonies, others at the centre. Pale violet and non-pigmented variants were seen in most strains.

Old cultures (4–7 days at 25°) showed colonies about 5 mm. in diameter; generally those of mesophils were dark violet and flat with lobate edge and those of psychrophils were low convex, sometimes showing concentric violet zones. The degree of pigmentation and of gelatinous consistency on nutrient agar (7 days at 25°) is shown in Table 2. Gelatinous strains may show non-gelatinous variants, as noted by Corpe (1953).

*Corynebacterium viscosum* and *Pseudomonas iodina* showed round greyish convex smooth colonies which were semi-opaque. Colonies of *C. viscosum* were non-pigmented on sugar-free media and were moderately viscid. *Pseudomonas iodina* sometimes developed iodine-like dark crystals of iodinin after some days at 20–25°. Strain TI produced colonies like those of smooth mesophils, but of a pale yellow colour and slightly viscid.

*Smell.* Aerobic plate cultures of mesophils gave an odour of hydrogen cyanide and ammonia. The psychrophils had a characteristic sour odour.

*Gelatin plates* (20°). The colonies of *Chromobacterium* varied from round, entire low convex shiny colonies to flat colonies with undulant surface and lobate or effuse edge. The colonies of most mesophils sank into cups of liquefied gelatin in a few days. Most psychrophils showed a little liquefaction in 5–7 days but rarely earlier. Deep colonies were lenticular or round, often with a mulberry-like internal structure. *Corynebacterium viscosum* and *Pseudomonas iodina* gave round convex entire smooth colonies, rather opaque. Those of the latter rapidly sank into the medium. Strain TI gave low convex round colonies, slowly sinking into the gelatin.

*Nutrient agar slopes.* Most cultures of *Chromobacterium* produced a smooth shiny violet growth, slightly raised with a finely lobate edge, but rough strains showed a dull or copper-beaten surface and irregular edge. Gelatinous growth and pigmentation were well developed after 7 days. *Corynebacterium viscosum* and *Pseudomonas iodina* gave semi-opaque shiny grey growth, and strain TI a pale yellow smooth growth.

*Gelatin stab* (20°). Most of the mesophils showed a filiform colourless growth along the stab, with a round pigmented button of surface growth. Liquefaction occurred in a few days; it was infundibuliform or napiform, never stratiform. After 14 days the top centimetre was usually entirely liquefied. The psychrophils gave a button of surface growth but scanty or no growth in the stab; liquefaction was very slow—generally none after 14 days, though strains DA, IN and EA showed slight crateriform liquefaction. The growth

Table 2. *Metabolic characteristics*

Strain	Pigment (7 days)	Gelatinous growth (7 days)	Highest growth temperature	Lowest growth temperature	Anaerobic growth (4 days)	Alkalinity on Simmons's medium (2 days)
Mesophils						
FH	++	—	44°	10°	+	+
MK	+++	—	37°	10°	+	±
BH	+++	—	44°	16°	+	—
BN	+++	—	44°	16°	+	±
AM	++	—	37°	10°	+	+
MW	++	—	37°	10°	+	±
TV	+	—	37°	10°	+	+
LG	+++	—	44°	10°	+	—
SL	+++	—	37°	10°	+	±
RV	++	—	37°	10°	+	±
DK	+++	—	37°	16°	+	±
TA	++	—	37°	10°	+	+
TB	++	—	37°	10°	+	+
PT	+++	—	37°	10°	+	±
LW	++	—	37°	10°	±	±
SH	++	—	37°	16°	±	±
RT	+++	—	37°	16°	±	—
MH	+++	—	37°	10°	±	±
Psychrophils						
NT	+	+++	25°	2°	—	+
EA	+	+++	30°	4°	—	+
EB	+	+	30°	2°	—	+
EC	+	+	30°	2°	—	+
GA	+	—	35°	2°	±	+
DA	++	—	30°	2°	—	+
NC	++	+	30°	2°	—	+
MA	++	+	30°	2°	—	+
MB	+	++	30°	2°	—	+
MC	++	+	30°	2°	±	+
HA	++	++	30°	2°	—	+
HB	++	++	30°	2°	—	+
HC	+++	+++	25°	2°	—	+
HD	++	+	30°	2°	—	+
HE	+++	++	30°	2°	—	+
HF	+	++	30°	2°	±	+
IN	+	+++	35°	2°	±	+
CA	+++	+++	30°	2°	—	+
PB	++	—	30°	2°	±	+
RU	++	++	30°	2°	—	+
Other bacteria						
<i>C. viscosum</i> GR		+	37°	10°	±	—
		(very viscid)				
<i>P. iodina</i> TE		—	37°	10°	—	—
<i>C. ianthinum</i> TI		—	37°	10°	±	+
		(viscid)				

*Symbols.* Pigment = + to +++ = increasing degrees of violet pigmentation. Gelatinous growth = + to +++ = increasing degrees of jelly-like consistency. Anaerobic growth: — = no growth visible with a lens; ± = growth visible with a lens; + = growth easily visible. Alkalinity on Simmons's medium: — = no change (c. pH 6.9); ± = slight alkalinity (c. pH 7.2); + = marked alkalinity (c. pH 7.5).



formed a thick pellicle on the liquefied gelatin and was well pigmented. *Corynebacterium viscosum* and *Pseudomonas iodina* gave rapid stratiform liquefaction with scanty growth in the stab and scanty pellicle without pigment. Strain TI showed moderate napiform liquefaction with yellow pellicle and a filiform growth in the stab.

*Nutrient broth.* Almost all strains gave a violet pellicle when the medium was not disturbed. This pellicle was fragile in the mesophils and thick in the gelatinous psychrophils. When the pellicle sinks, a very characteristic violet ring of growth, which adheres to the glass, remains at the surface; it is thin and powdery, except for the gelatinous psychrophils, where it is thick, viscous and shows hanging fronds, even in those which were only slightly gelatinous on agar. The fluid was clear with auto-agglutinable or gelatinous strains, and turbid with the others. A deposit was present and had the consistency of the pellicle; it might remain violet or become pale. The fluid itself was stained slightly violet by the heavily pigmented strains. Being strict aerobes, the psychrophils grew poorly in broth. *Corynebacterium viscosum* and *Pseudomonas iodina* grew poorly without pellicle. Strain TI gave a yellow ring, with turbidity and deposit.

*Blood agar plates.* The colonial appearances were similar to those on nutrient agar, but growth was better and isolated colonies grew readily. Rapid haemolysis is characteristic of mesophils (see below).

*Löffler's serum slopes.* Growth was good, soft and shiny. The mesophils generally produced moderate digestion in 2 weeks, leading to collapse of the medium. The psychrophils usually gave little digestion in this time (see Table 4). Pigmentation was erratic. *Corynebacterium viscosum* and *Pseudomonas iodina* gave greyish growth without digestion. Strain TI caused rapid digestion.

*Potato slopes.* Growth on potato has been used as a differential test by Cruess-Callaghan & Gorman (1933). In my hands it was not useful because it was difficult to assess the amount of growth when the degree of pigmentation varied widely. All strains grew, giving smooth undulant growth with a diffuse edge, often spreading. In the drier parts, flat stellate colonies might form. Violet pigmentation was heavy with mesophils. Psychrophils usually showed less pigment, brownish violet colours being the rule. Strains EB, EC, GA, MB, MC and HF gave yellowish growth, while strains NT, MA, and HE gave deep violet growth after 2 weeks. The gelatinous consistency of strains was not well marked on potato. *Corynebacterium viscosum* produced a slightly viscous cream-coloured growth, not spreading, with a sky-blue pigment after 10 days, which diffused across the potato. *Pseudomonas iodina* gave a raised moist grey growth without pigment. Strain TI gave a smooth growth which was reddish violet (diffusing slightly) after 7 days and brownish after 14 days.

### Resistance

*Heat resistance.* None of the organisms studied showed unusual resistance to heat; 5 ml. broth cultures were sterile after 30 min. at 56°. With *Chromobacterium* strains and strain TI loopfuls of broth seldom showed viable

organisms after 5 min. at 56° and never after 10 min. *Corynebacterium viscosum* and *Pseudomonas iodina* showed a few viable organisms in a loopful after 15 min. The difference reported by Bampton (1913) between *Bacillus violaceus* and *B. membranaceus amethystinus* was not confirmed; no strain was completely killed by 42.5° or 45° in 30 min. and those which showed fewest colonies after heating (strains MW, NT, TV, LW, RT, TA, TB, CA and RU) had no other distinctive features in common.

*Phenol resistance.* No strain of *Chromobacterium* nor strain TI showed viable organisms in 0.05 ml. after 10 min. in 1% (w/v) phenol, though an occasional strain showed viable organisms after 5 min. *Corynebacterium viscosum*, *Pseudomonas iodina*, *P. aeruginosa* (2 strains) and 'Bacillus prodigiosus' (2 strains) showed many surviving organisms after 10 min.

*Resistance to penicillin.* All strains of *Chromobacterium* and strain TI were resistant to 100 units benzylpenicillin/ml., but *Corynebacterium viscosum* and *Pseudomonas iodina* were sensitive to this concentration.

*Longevity of cultures.* Agar cultures of *Chromobacterium* strains usually died out in a week or two at 20°. Broth cultures survived for several weeks at 20°, while at 2° the psychrophils lived longer but the mesophils seemed to die out more rapidly. The alkalinity of old cultures may be responsible, since sloppy agar cultures (which become strongly alkaline only at the surface) survived longer. For transport through the post, agar stab cultures have been best. It is known that dried cultures also do not survive well (Rhodes, 1950); my experience has been similar. Good results, up to 1 year of storage, have been obtained by storing broth cultures at -78°. In these respects the organisms resemble the vibrios.

#### *Metabolism and nutrition*

*Anaerobic growth.* All the mesophils grew, but most psychrophils did not grow in 4 days (Table 2). No pigment was formed.

*Growth temperature.* The results are shown in Table 2, which gives the lowest and the highest of the temperatures used at which growth occurred. No strain grew both at 2° and at 37°. The optimum temperature was nearer the upper limit than the lower. Some mesophils (strains AM, MW, TA, TV, LW and MH) grew poorly (or failed to grow on very dry slopes) at 37°, but these did not resemble the psychrophils in other respects.

*pH Value for growth.* The behaviour was more or less uniform with all strains: growth took place on nutrient agar at pH 6 and 9, but not at pH 5 or pH 10; the optimum value was between pH 7 and 8. Tests with a few strains in broth showed that slow growth sometimes occurred at pH 5.2, but this did not appear to be of value in classification.

*Utilization of citrate as sole source of carbon.* It is possible that discrepancies may arise between results obtained with the Koser medium and the Simmons medium, since the former should in theory be free from growth factors, while the latter may contain growth factors from the agar. Therefore Koser's solution was made up to be growth-factor free as far as possible, while yeast extract was added to the Simmons medium (this amount of yeast extract gave scarcely visible growth and no change in pH value when the citrate was omitted).

All strains of *Chromobacterium* and strain TI grew slowly in Koser's medium in both tubes, and grew on Simmons's medium, turning it alkaline. With the latter there was considerable strain variation, the psychrophils turning it alkaline in 2 days, while some mesophils took 5 or 6 days. The differences did not correlate well with other features of the strains. *Corynebacterium viscosum* was negative in both media after 1 week. *Pseudomonas iodina* grew very slowly in both.

*Tolerance of sodium chloride.* Almost all strains grew well on 3% (w/v) NaCl agar, and failed to grow on 6.5% NaCl agar. There was often slow growth in 6.5% NaCl broth. Strain TI and *Corynebacterium viscosum* grew slightly on 6.5% NaCl agar, while *Pseudomonas iodina* grew luxuriantly.

*Catalase effect.* *Chromobacterium* strains, like *Pasteurella pestis*, are very sensitive to traces of hydrogen peroxide, as shown by the inability of single organisms to grow aerobically on nutrient agar, and the small number of separated colonies obtained when strains are plated out on this medium. The inhibition is overcome by blood, haematin or catalase (Sneath, 1955). The strains reported on by Sneath (1955) were all mesophils; but six psychrophils have since been examined and all show the effect.

*Production of hydrogen cyanide.* Aerobic plate cultures of all the mesophils smell of HCN and this can be demonstrated by indicator paper, most easily in cultures grown at 35–37°. The amount of HCN produced is considerable (Sneath *et al.* 1953), being more than that produced by *Pseudomonas aeruginosa* (Patty, 1921; Lorck, 1948) which does not give a positive reaction with the test-paper. Clawson & Young (1913) first reported on production of HCN by *Chromobacterium* but did not mention the amount produced. By growing a typical mesophil (MK) in flat bottles the gas was identified by the Prussian blue reaction. Anaerobic cultures produce little HCN. The psychrophils, 'Bacillus prodigiosus', strain TI, *Corynebacterium viscosum* and *Pseudomonas iodina* gave negative reactions with the indicator paper. In flat bottles strain HB was negative (2 and 4 days growth at 25°). Despite the production of cyanide, growth is scanty in the medium of Møller (1954) with all strains in 3 days at 25°.

*Products of attack upon glucose.* The main products detected were as follows: strain MK, which ferments glucose anaerobically, produced acetic acid, *n*-butyric acid, probably some succinic and lactic acids and an unidentified non-volatile acid; strain LW (which oxidizes glucose) produced acetic and succinic acids; strain HB (which oxidizes glucose) produced a little acetic, succinic and lactic acids and several unidentified non-volatile acids. There was probably some gluconic acid in all these cultures.

*Pigment formation.* All the strains of *Chromobacterium* produced on nutrient agar a violet pigment which was non-diffusing (slight diffusion may occur in old cultures), soluble in ethanol, and which became green and then red-brown with caustic potash. Both mesophils and psychrophils appear to produce the same pigment, violacein. Pigments from two mesophils, MK and RV, and from two psychrophils, NT and HB, were examined with a range of common reagents and by spectrophotometry. They behaved identically and in general agreement with the reports in the literature.



On treating ethanolic solutions with equal volumes of the following reagents, the corresponding colour changes were noted: 10% KOH: green, turning reddish and then brown in a few minutes; 50% HNO<sub>3</sub>: yellowish orange; 50% H<sub>2</sub>SO<sub>4</sub>: emerald green, stable for many hours; glacial acetic acid: blue, stable; 2M-HCl: blue-green, turbid, stable; H<sub>2</sub>O<sub>2</sub> (10 vol. %): remains violet, stable at 20°, decolorized at 80°; bromine water: decolorized; 5M-NH<sub>4</sub>OH: sapphire blue, fading to colourless; 0.1M-FeCl<sub>3</sub>: pale yellow. A solution of the pigment reduced with 25% acetic acid and zinc dust slowly decolorized; with 0.5% NaNO<sub>2</sub> in 10% acetic acid it becomes pale brown and turbid; it did not give a red colour with the Böhme indole reagents.

Samples of violacein from strains RV and NT were purified by the method of Strong (1944) and recrystallized from pyridine + chloroform. On heating, the pigment decomposed without melting. The absorption spectrum in 96% ethanol in water is shown in Fig. 1. Both samples gave identical curves, and the wavelengths of the observed maxima and minima, with the Specific Extinction Coefficients (optical density of 1 cm. thickness of a solution containing 1 g./l.) were:

208 mμ.	247 mμ.	257.5 mμ.	339 mμ.	374 mμ.	431 mμ.	579 mμ.
110	51.3	54.9	13.6	17.8	7.4	65.4

The extinctions are somewhat higher than those found by Ehrismann & Noethling (1936). The purified pigments were also examined in 10% (v/v) H<sub>2</sub>SO<sub>4</sub> (sp.gr. 1.84) in 96% ethanol; the green solution was stable for some hours. Both samples gave identical curves. The observed maxima and minima (see Fig. 1) and Specific Extinction Coefficients were:

235 mμ.	250 mμ.	269 mμ.	280 mμ.	295 mμ.	301 mμ.
40.4	52.2	36.8	41.6	29.1	29.5
337 mμ.	413 mμ.	502 mμ.	653 mμ.	659 mμ.	699 mμ.
6.2	21.8	8.5	73.4	73.0	102.7

The infrared absorption spectra of the two crystalline pigment samples (kindly determined by Dr R. K. Callow) were virtually identical, and the 'finger-print region' of the spectrum is shown in Fig. 2.

Crude pigments from several mesophils and psychrophils (extracted from the organisms with ethanol) contained impurities which distorted the absorption curves in the ultraviolet region, but the curves were otherwise similar to those of purified samples, and were in good agreement with the curve published by Gilman (1953) for *Chromobacterium violaceum*. An example is shown in Fig. 1.

The dry pigment was almost insoluble in water, chloroform or benzene; it was readily soluble in ethanol or acetone and in ethanol + water and acetone + water mixtures, giving violet solutions. In ether it was very slightly soluble, the solution having a more reddish tone than in ethanol. Violacein has probably a hydroxyindolyl-oxindolyl-pyrryl-methene structure (Beer, Jennings & Robertson, 1954) and the infrared absorption spectrum is compatible with such a structure. Non-pigmented variants often became tinged with yellow after some days; this second pigment was not investigated.

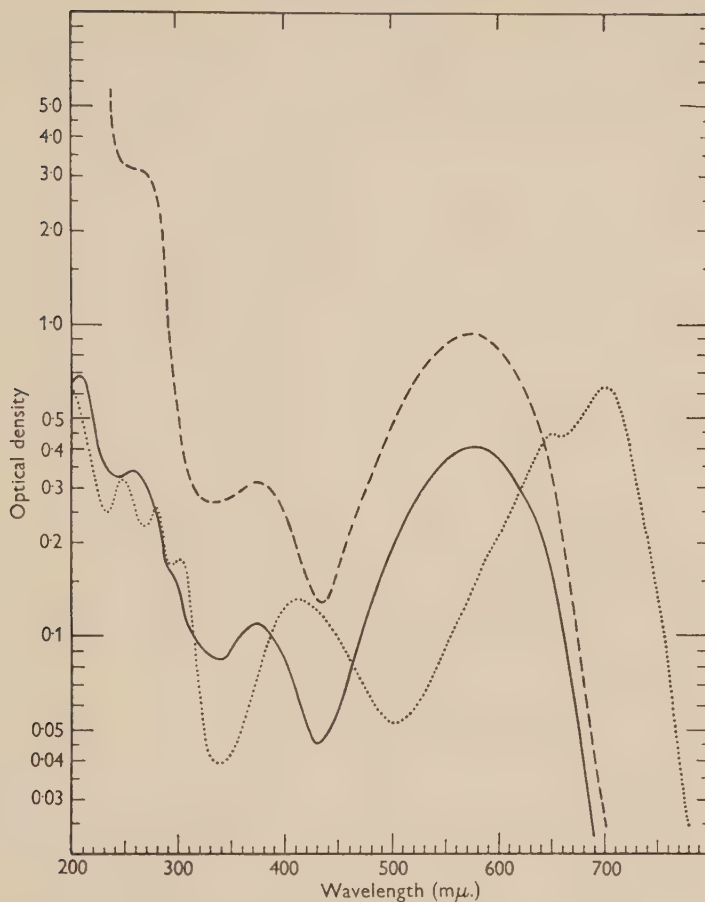


Fig. 1. Absorption spectra of violacein (in  $\frac{1}{2}$  cm. cells). —, Ethanolic solution of crystalline violacein (14.0 mg./l.); . . . ., crystalline violacein, 14.0 mg./l. in 10% (v/v) sulphuric acid in 96% ethanol; - - - -, crude violacein (from strain HB) in ethanol.

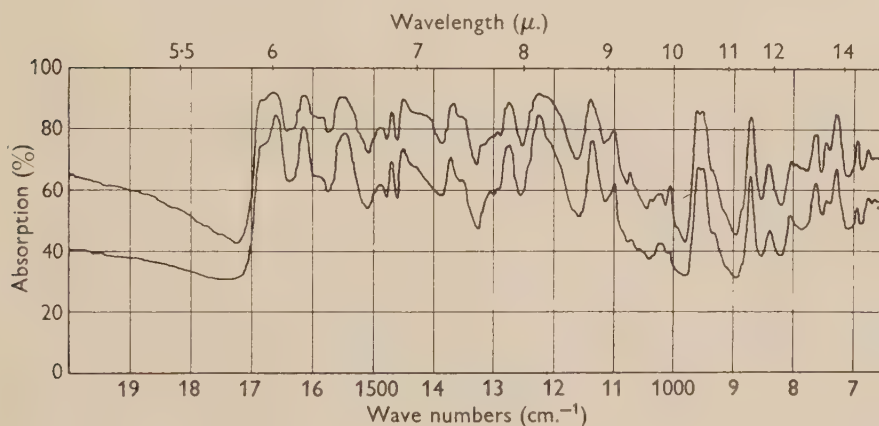


Fig. 2. Infrared spectrogram of violacein (in KCl disks). Upper curve: violacein from strain RV; lower curve: violacein from strain NT. The concentration of pigment was slightly different in the two experiments.

*Corynebacterium viscosum* did not produce pigment on nutrient or peptone agar. Upon glucose, sucrose or mannitol peptone agar it produced after 1 day at 25° a small amount of a green diffusing pigment, and after several days a large amount of a diffusing blue-violet pigment. The blue-violet pigment from sucrose peptone agar (extracted with water) in 0.5M-potassium phosphate

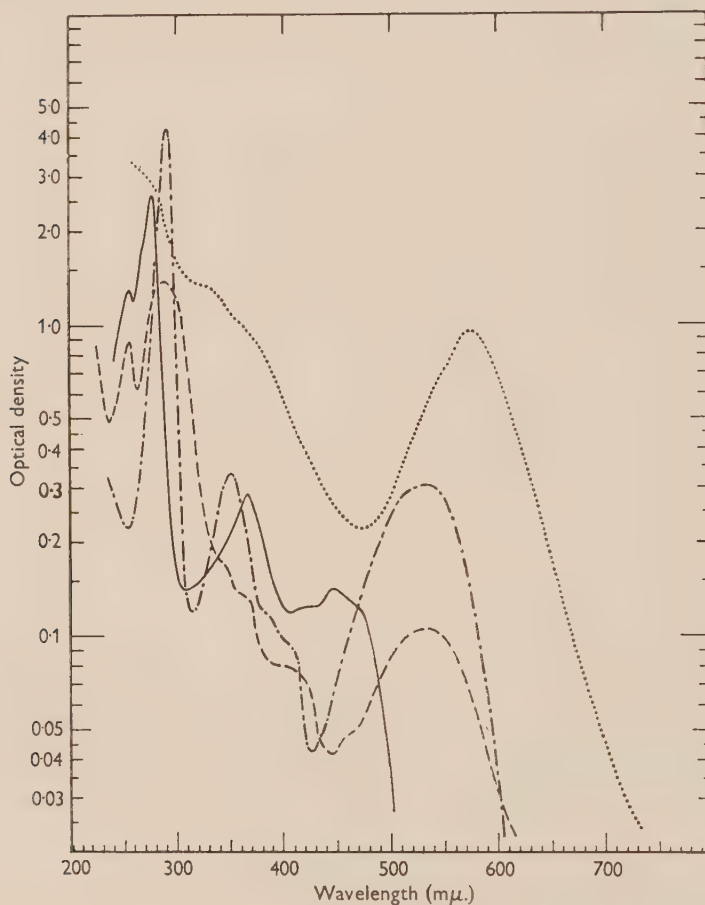


Fig. 3. Absorption spectra of pigments of *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI. —, pigment of strain TI in acid chloroform; ---, pigment of strain TI in 0.1M-NaOH; . . . ., pigment of *C. viscosum* in buffer (pH 7.0); - · - · - ·, iodinin in chloroform (9.4 mg./l. in  $\frac{1}{2}$  cm. cell).

buffer (pH 7.0) showed an absorption peak at 574 mμ. and maximal transmission at 472 mμ. (Fig. 3); in 0.5M-phosphate buffer (pH 4.2) these were at 566 and 468 mμ., respectively. At both pH values there were shoulders or small peaks at about 275 and 330 mμ. Gilman (1953) gave a curve similar to that found at pH 4.2. The pigment became reddish in 0.1M-HCl, and blue or blue-green in 0.1M-NaOH. Addition of saturated aqueous  $\text{MgSO}_4$  turned solutions purple.



*Pseudomonas iodina* produced the characteristic iodine-like crystals described by Davis (1939); these were insoluble in water, but soluble in warm ethanol and in chloroform, giving reddish purple solutions. The pigment, iodinin, is 1:6-dihydroxy-phenazine 5:10-dioxide (International convention) melting point 236° dec. (Clemo & Daglish, 1950). The crystals were erratic in occurrence, only some batches of nutrient and blood agar being suitable; peptone agar was more reliable. Gilman (1953) however reported the pigment as brownish and water-soluble, but his absorption curve is almost identical with that of iodinin in chloroform. Iodinin from strain RE recrystallized twice from chloroform had melting point 235° (dec.). The absorption spectrum of a solution in chloroform is shown in Fig. 3. There are some differences from the curve given by Clemo & McIlwain (1938), but a sample of their pigment (kindly provided by Professor McIlwain) had an absorption spectrum identical with that of my sample. The Molecular Extinction Coefficients observed at the maxima and minima were:

255 m $\mu$ .	291 m $\mu$ .	317 m $\mu$ .	352 m $\mu$ .	427 m $\mu$ .	534 m $\mu$ .
7,270	140,000	3,900	11,000	1,350	10,100

Strain TI appeared to produce several pigments. Upon nutrient agar, peptone agar or glucose peptone agar it produced only a pale yellow, poorly diffusing pigment. Upon potato and on mannitol and sucrose peptone agar it produced a poorly diffusing red-violet pigment at 20° or 25° but little at 37°. In old peptone water + carbohydrate tubes a dark red pigment sometimes formed in the pellicle. This pigment was almost insoluble in water, ethanol, chloroform or benzene, but dissolved in 0.1M-HCl, and turned yellow; it could then be extracted with chloroform. The substance turned red-violet and passed into the aqueous phase on shaking the chloroform with 0.1M-NaOH. It is possible that the three pigments are closely related, since the yellow one became mauve with alkalis and the mauve one became yellow with acids. Gilman (1953) used an ethanolic extract of organisms grown on nutrient agar for his studies; his extract evidently contained some yellow pigment.

The mauve pigment from strain TI grown on mannitol peptone agar was extracted with chloroform after scraping the growth into 0.1M-HCl, and the chloroform extracted with 0.1M-NaOH. The alkaline solution was again acidified with HCl and the process repeated twice. This partly purified pigment was yellow when extracted with chloroform from acid, and was stable for some hours; the absorption spectrum showed absorption maxima at 277, 367 and 447 m $\mu$ . and minima at 307 and 406 m $\mu$ . When extracted into 0.1M-NaOH, the unstable mauve solution showed maxima at 255, 286 and 536 m $\mu$ . and minima at 262 and 446 m $\mu$ . with shoulders at about 360 and 400 m $\mu$ . (Fig. 3).

*Growth requirements.* Eight mesophils and two psychrophils grew in ammonium + lactate medium and could be subcultured in this. Although growth was much increased by addition of casein hydrolysate, the results suggest that there is no absolute requirement for amino acids or growth factors, despite the report of Linardos & Cleverdon (1955) that all strains require organic nitrogen. The results with Koser's citrate also support this.

*Carbohydrate fermentation*

It was considered most useful to study fermentation reactions in peptone water for comparison with earlier records, and because of their wide use. Work with chemically-defined media will clearly be valuable. None of the strains of *Chromobacterium* produced gas. Most of the psychrophils showed only slight acid production, and there were therefore many doubtful reactions. No acid was produced by any strain from the following compounds: cellobiose, raffinose, melezitose, *m*-erythritol, dulcitol, inositol, adonitol, lactose, rhamnose,  $\alpha$ -melibiose. Doubtful reactions were often seen with the following carbohydrates, but were not sufficiently constant to be useful: salicin, D(+)xylose, L(+)arabinose, mannitol, aesculin, inulin, galactose, sorbose, glycerol, sorbitol, maltose (the last four most often with the mesophils). The carbohydrates which showed definite acidity with some strains are shown in Table 3, in which are also shown the results of testing a few carbohydrates in the medium of Hugh & Leifson.

It is seen that the mesophils attacked glucose, fructose, mannose and trehalose promptly. Sucrose and the triad dextrin, glycogen and starch were attacked, often late, by some strains, and it is possible that this was due to the occurrence of mutants, since in strain BN a variant which fermented starch promptly was isolated by plating the culture on to starch plates. Most of the mesophils were also tested at 37°; the reactions were substantially the same, but acid production was more rapid and more marked. The psychrophils often produced slight acidity from some sugars. Trehalose (in either medium) was the most useful sugar for distinguishing the two groups. Aesculin also differentiated the groups, but since those strains which split it did not ferment glucose strongly, it must be used as described below. Unlike the mesophils the psychrophils did not ferment glucose anaerobically. The four mesophils which did not ferment glucose were old stock strains, and they may well be loss-variants: they also attacked mannose slowly or not at all. *Corynebacterium viscosum* and *Pseudomonas iodina* gave no definite acidity in peptone water + carbohydrates, while strain TI gave slight acidity only in glucose or galactose. *P. iodina* appeared not to attack glucose aerobically or anaerobically.

*Results of biochemical tests*

*Litmus milk.* The results were variable. Slight acidity was more common with mesophils, but moderate alkalinity sometimes occurred after a few days. Some strains produced initial alkalinity and late acidity. The mesophilic strains usually peptonized the casein after some days, and often gave a small clot. The psychrophils commonly produced a large 'rennin' clot and seldom showed marked peptonization. Some pigment was usually formed, as a violet ring of growth or in a pellicle which was sometimes thick with gelatinous strains. About half the psychrophils bleached the litmus. Peptonization is the most useful feature, and parallels the results on milk plates (Table 4). *Corynebacterium viscosum* and strain TI produced slight acidity and slight peptonization. *Pseudomonas iodina* produced alkalinity and a clot.

Table 3. *Fermentation of carbohydrates*

Strain	Reactions in peptone water carbohydrates								Reactions in Hugh & Leifson's medium		
	Glucose	Fructose	Mannose	Trehalose*	Sucrose	Glycogen	Dextrin	Starch	Glucose	Fructose	Mannose
Mesophils											
FH	A	A	A	A	—	—	—	—	F	F	F
MK	A	A	A	A	—	—	—	—	F	F	F
BH	A	A	A	A	Al	Al	Al	al	F	F	F
BN	A	A	A	A	—	Al	Al	Al	F	F	F
AM	A	A	A	A	—	—	—	—	F	F	F
MW	A	A	A	A	—	—	—	—	F	F	F
TV	A	A	A	A	—	al	al	al	F	F	F
LG	A	A	A	A	A	Al	Al	Al	F	F	F
SL	A	A	A	A	A	—	—	—	F	F	F
RV	A	A	A	A	A	—	—	—	F	F	F
DK	A	A	A	A	—	—	—	—	F	F	F
TA	A	A	A	A	—	—	—	—	F	F	F
TB	A	A	A	A	—	—	—	—	F	F	F
PT	A	A	A	A	—	Al	Al	Al	F	F	F
LW	A	A	—	A	—	—	—	—	O	O	—
SH	A	A	—	A	—	—	—	—	O	O	—
RT	A	A	a	A	—	—	—	—	O	O	O
MH	A	A	a	a	a	—	—	—	O	O	Ol
Psychrophils											
NT	a	—	—	—	—	—	—	—	O	O	O
EA	A	A	al	—	—	—	—	—	O	O	O
EB	a	a	a	—	—	—	—	—	O	O	O
EC	—	—	—	—	—	—	—	—	O	O	O
GA	—	—	—	—	—	—	—	—	O	O	O
DA	a	—	—	—	—	—	—	—	O	O	O
NC	—	—	—	—	—	—	—	—	O	O	O
MA	a	al	—	—	—	—	—	—	O	O	O
MB	—	al	—	—	—	—	—	—	O	O	O
MC	—	—	—	—	—	—	—	—	O	O	O
HA	—	—	—	—	—	—	—	—	O	O	O
HB	—	—	—	—	—	—	—	—	O	O	O
HC	—	a	—	—	—	—	—	—	O	O	O
HD	—	—	—	—	—	—	—	—	O	O	O
HE	—	a	—	—	—	—	—	—	O	O	O
HF	—	—	—	—	—	—	—	—	O	O	O
IN	—	—	—	—	—	—	—	—	O	O	O
CA	—	—	—	—	—	—	—	—	O	O	O
PB	—	—	—	—	—	—	—	—	O	O	O
RU	—	—	—	—	—	—	—	—	O	O	O
Other bacteria											
<i>C. viscosum</i>	—	—	—	—	—	—	—	—	Fl	O	O
GR	—	—	—	—	—	—	—	—	—	—	—
<i>P. iodina</i>	—	—	—	—	—	—	—	—	—	—	—
TE	—	—	—	—	—	—	—	—	—	—	—
<i>C. ianthinum</i>	a	—	—	—	—	—	—	—	O	O	O
TI	—	—	—	—	—	—	—	—	—	—	—

A = marked acidity; a = slight acidity; l = late (in 7-14 days); F = fermentative attack (acidity anaerobically); O = oxidative attack (acidity only aerobically); — = no acidity or doubtful result.

\* Trehalose in aerobic Hugh & Liefson medium also gave acidity with all mesophils but no acidity with any psychrophil.



Table 4. *Biochemical reactions*

Incubation temperature 25°.									
Strain	Aesculin hydrolysis (4 days)	Haemolysis horse blood agar (2 days)	Egg-yolk reaction (2 days)	Reduction of		Gelatin hydrolysis (4 days)	Casein hydrolysis (2 days)	Liquefaction of serum (14 days)	Gluconate test (4 days)
				nitrate to nitrite (5 days)	Destruction of nitrite (5 days)				
Mesophils									
FH	—	+	+	—	—	+	++	++	—
MK	—	++	++	+	+	++	++	++	—
BH	—	++	++	+	+	++	++	++	—
BN	—	++	++	+	+	++	++	++	—
AM	—	—	++	+	±	++	+	++	—
MW	—	++	++	+	+	++	++	++	±
TV	—	++	++	+	++	++	++	++	—
LG	—	++	++	+	+	++	++	++	—
SL	—	++	++	+	—	++	++	++	—
RV	—	++	++	+	+	++	++	++	—
DK	—	++	++	+	+	++	++	++	—
TA	—	++	++	+	++	++	++	++	—
TB	—	++	++	+	+	++	++	++	—
PT	—	±	+	+	+	++	++	+	—
LW	—	±	+	+	±	+	++	++	—
SH	—	±	+	+	±	+	++	++	—
RT	—	±	++	+	—	+	+	++	—
MH	—	+	++	+	+	+	++	+	—
Psychrophils									
NT	+	—	—	—	—	—	±	±	±
EA	+	±	—	+	++	±	—	—	+++
EB	+	—	—	+	±	±	—	+	+++
EC	+	—	—	+	+	—	—	—	+
GA	+	—	—	+	±	—	—	—	—
DA	+	—	—	+	—	±	—	—	—
NC	+	—	—	+	—	—	—	±	—
MA	+	—	—	+	±	—	—	—	—
MB	+	—	—	—	+	±	±	±	—
MC	+	—	—	+	++	±	+	—	—
HA	+	—	—	+	+	±	±	—	+++
HB	+	—	—	+	+	—	—	±	+++
HC	+	—	—	+	+	—	—	—	++
HD	+	—	—	+	+	—	—	+	—
HE	+	—	—	+	+	—	±	—	++
HF	+	±	—	+	+	±	—	+	—
IN	+	—	—	+	+	—	—	—	—
CA	+	—	—	+	+	—	—	—	—
PB	+	—	—	+	+	—	—	—	—
RU	+	—	—	+	+	—	±	—	—
Other bacteria									
GR*	+	—	—	+	±	+	+	—	—
TE†	—	++	—	—	—	+	—	—	—
RE‡	.	.	.	+	—	.	.	.	.
TI§	+	—	+	—	—	+	+	+	+++

*Symbols.* Aesculin hydrolysis (plate test): —=no browning; +=wide zone of browning. Haemolysis, casein hydrolysis and gelatin hydrolysis: —=no clearing; ±=clearing only beneath the growth; +=zone of *c.* 2 mm. clearing; ++=zone of *c.* 5 mm. clearing. Egg-yolk reaction: —=no opacity; +=opaque zone of *c.* 2 mm.; ++=opaque zone of *c.* 5 mm. Reduction of nitrate: —=no colour; +=detectable pink colour. Destruction of nitrite: —=strong colour; ±=faint colour; +=no colour; ++=no colour after only 24 hr. incubation. Liquefaction of serum (Löffler's slopes): —=no change; ±=partial clearing of medium; +=furlowing of medium; ++=collapse of slope. Gluconate test: —=no reduction of Benedict's solution on heating; ± to +++=increasing reduction on heating, from a green colour to a deep orange precipitate. Strain RE: . =not tested.

\* *C. viscosum.*† *P. iodina.*‡ *C. iodinum.*§ *C. ianthinum.*

*Indole, ammonia, methyl red (MR) and Voges-Proskauer (VP) tests.* All strains of *Chromobacterium* were indole-negative. Most strains were strongly ammonia-positive, a few weakly positive, and were all MR-negative, except that a few mesophils gave a doubtful reaction. All strains were VP-negative, the few doubtful reactions being probably due to the effect of the alkali on the pigment, which turned green and then brown. *Corynebacterium viscosum* and *Pseudomonas iodina* and strain TI were indole-negative, MR-negative and VP-negative; the first two were  $\text{NH}_3$ -negative, the last  $\text{NH}_3$ -positive.

*H<sub>2</sub>S production and reduction of methylene blue.* No strain produced abundant H<sub>2</sub>S, though the mesophils sometimes produced a trace when incubated at 37°. Most of the mesophils reduced methylene blue, while most of the psychrophils did not, or gave a doubtful result. *Corynebacterium viscosum* was negative in both tests; *Pseudomonas iodina* and strain TI were H<sub>2</sub>S-negative but reduced methylene blue weakly.

*Reduction of nitrate and destruction of nitrite.* The majority of strains of *Chromobacterium* gave positive reactions in both tests (Table 4). Several mesophilic strains, e.g. strain TV, destroyed nitrates and nitrites so actively, particularly at 37°, that the test for nitrite was negative on the fifth day. No bubbles of nitrogen gas were noted in the tubes. Nitrite was usually not completely destroyed after 1 day. The negative strains are probably loss-variants, which may occur in nature. In strain SH, a variant which was negative in both tests occurred spontaneously.

*Catalase, urease and phosphatase.* All strains of *Chromobacterium* were catalase-positive, though sometimes weakly so. The method of testing by pouring hydrogen peroxide on to a slope culture often gave doubtful results. All strains were urease-negative and moderately to strongly phosphatase-positive. *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI were also catalase- and phosphatase-positive but urease-negative.

*Hydrolysis of aesculin.* The psychrophilic strains hydrolysed aesculin, producing a dark brown colour, while the mesophils did not (Table 4). Concordant results were obtained with the liquid and solid media, but the latter gave more rapid results, showing a brown zone in 24 hr. *Corynebacterium viscosum* was positive on plates but doubtful in the liquid medium.

*Hydrolysis of casein, gelatin and starch.* Zones of clearing due to hydrolysis of casein were produced in 2 days by most of the mesophilic strains but not by most of the psychrophils (Table 4). After longer periods many psychrophils were positive, and generally showed clearing under the growth. In the gelatin plates after 4 days the mesophils usually showed a wide zone of clearing, while the psychrophils produced none or only clearing beneath the growth (Table 4). The results run almost parallel to those for liquefaction in gelatin stabs. None of the strains studied here completely hydrolysed the starch in 4 days, but strains PT and LG gave partial hydrolysis beneath the growth.

*Haemolysis on horse blood agar and egg-yolk reaction.* The mesophils generally gave a wide zone of haemolysis in 2 days (Table 4), which was slight or absent with the psychrophils, though these sometimes showed a little haemolysis after 4 days of growth. The haemolysis is not true  $\beta$ -haemolysis, being

a partial clearing with a diffuse edge; probably much of it is due to the lecithinase.

Upon egg-yolk plates all the mesophils gave positive reactions (presumably due to lecithinase), usually in 2 days and well marked in 4 days, while the psychrophils remained negative (Table 4).

*Malonate, phenylpyruvate, gluconate and arylsulphatase tests.* None of the strains of *Chromobacterium* studied gave positive reactions for phenylpyruvic acid or arylsulphatase. Malonate was utilized only by strain CA in 2 days. The gluconate test gave unexpected results. A few strains (see Table 4) produced a substance which reduced Benedict's solution, but unlike the *Pseudomonas* cultures, or 2-keto-D-gluconic acid, which only reduce the reagent on heating, the *Chromobacterium* cultures reduced the reagent in the cold in a few minutes. Some preliminary tests on these cultures showed that the reducing substance immediately reduced phenol blue ( $E'_0 = +0.224$  V.) but not methylene blue ( $E'_0 = +0.011$  V.) at pH 7.0; this suggests a structure similar to ascorbic acid. *Corynebacterium viscosum*, *Pseudomonas iodina* and strain TI also gave negative reactions for phenylpyruvate arylsulphatase and malonate utilization.

#### DISCUSSION

The name *Chromobacterium* is used here in a narrow sense. The name was used by Topley & Wilson (1929) as one of convenience, to include red and yellow chromogens such as 'Bacillus prodigiosus' (*Serratia marcescens*); this organism appears to be a typical member of the Enterobacteriaceae, and if unpigmented, many strains could scarcely be distinguished from some organisms of the coli-aerogenes group. *Pseudomonas* also seems to be generically distinct. I agree with Gilman (1953) that *Corynebacterium viscosum* and *Pseudomonas iodina* cannot be retained in the genus. They are both Gram-positive non-motile rods, in morphology similar to diphtheroids, and they do not produce violacein: they would not be placed in *Chromobacterium* if they did not produce pigment. Strain TI is not the *C. ianthinum* of older authors. It does not produce violacein, and appears to be a pseudomonad and it should be excluded from the genus.

The results reported here show that the genus *Chromobacterium* can be divided into two main groups. Features which, apart from the violet pigment, violacein, are unusual in bacteria are the mixed type of flagellation, the poor survival of cultures on storage and the sensitivity to inhibition by peroxide. Despite the considerable differences in other characteristics (e.g. method of attack upon carbohydrates) and the absence of strains which are clearly intermediate between the two groups these unusual features support the thesis that they all belong to one genus. The systematic position of this genus is uncertain; it possesses many characteristics of the Pseudomonadaceae and some characteristics of the Enterobacteriaceae. It may be closely allied to *Vibrio*. In practice it is the production of violacein which is the main distinguishing feature of *Chromobacterium*. This is unsatisfactory, since unpigmented strains which may be common in nature are probably never assigned to the genus; there may be non-chromogenic species which should be placed in the genus.



The two groups distinguished within the genus have a large number of correlated characteristics, and may be regarded as two species or biotypes. One can distinguish a subgroup in the mesophils, represented by the four strains which do not ferment glucose anaerobically. Since, however, most of the other aberrant features of these four strains (e.g. anaerobic growth, attack on other carbohydrates, storage of fat) may perhaps depend on one or two blocks in the carbohydrate metabolism of these strains, and since all my examples are old stock cultures which may have lost some characteristics, it would seem wise not to regard them as a distinct group without further studies. Such loss

Table 5. *Differential characteristics of mesophilic strains (18) and psychrophilic strains (20) of Chromobacterium*

Characteristic	Mesophils	Psychrophils
Growth at 37°	Occurs	Absent
Growth at 4°	Absent	Occurs
Relative size of organisms	Usually small	Usually large
Fat in organisms	Usually abundant	Usually scanty
Proteolysis	Marked	Slight
Haemolysis	Marked	Slight
Anaerobic growth	Usually good	Scanty or absent
Utilization of citrate	Often slow	Rapid
Production of HCN	Abundant	Not detected
Egg-yolk reaction	Positive	Negative
Attack on glucose	Usually fermentative	Oxidative
Acid from trehalose	Positive	Negative
Hydrolysis of aesculin	Negative	Positive

is not unknown in other genera, e.g. *Pfeifferella pseudomallei* (see *Topley and Wilson's Principles*, 1946, p. 487). The features closely correlated with growth temperature are shown in Table 5. Of these, a positive egg-yolk reaction, aesculin hydrolysis, HCN production and acid from trehalose show almost absolute correlation. The following features show some correlation: some mesophils are naturally pathogenic for mammals, they do not give gelatinous growth, give good pigment on potato and are usually methylene blue positive; the psychrophils are not naturally pathogenic, are often gelatinous, often pigment poorly on potato and are usually methylene blue negative. The following characteristics are either not correlated with growth temperature (or with each other), or insufficient evidence is yet available: source from soil or water, country of source, type of flagellation, degree of pigmentation, degree of growth on potato, reaction in litmus milk, acid from sucrose and starch, gluconate reaction, attack on nitrates and nitrites, heat resistance at 45°, colonial form and presence of metachromatic granules: none of these seems suitable as a differential characteristic.

### Nomenclature

The nomenclature of the genus is confused, and will require action by the International Committee on Bacteriological Nomenclature to make it legitimate. Proposals have been submitted to the Committee for the conservation

of the generic name *Chromobacterium*, with the designation of a type species and type strain; these proposals have been published (Sneath, 1956b). The position will be fully dealt with there, and only a summary is given below.

Bergonzini (1879) used the name *Cromobacterium* for those members of the genus *Bacterium* which produced pigment. He recognized four species, three of which are now classified as *Pseudomonas* spp. while the fourth is unrecognizable. Later he described a new species which he named *Cromobacterium violaceum* (Bergonzini, 1880). Buchanan (1918) revived the name (with emended spelling) as that of a genus, and defined it in terms which virtually restricted it to the group of bacteria studied in this paper. Buchanan designated the type species as *Chromobacterium violaceum* Bergonzini. This designation of the type is illegitimate under Rule 9 (2) c2 of the revised International Bacteriological Code of Nomenclature (Editorial Board, 1953 (p. 42); International Committee, 1953 (Minute 6, p. 158)). In addition, it is not possible to recognize the species of Bergonzini's *C. violaceum* from his description, although it was almost certainly a member of the genus as defined here. It would be confusing and inconvenient to create a new generic name, and it would be better to conserve the name, basing it on a new type culture.

There are, in my opinion, two species within the genus, corresponding to the mesophilic and psychrophilic groups described in this paper. Many of the early descriptions of micro-organisms of this genus are unrecognizable as to species, since almost the only features described by early authors which allow recognition of the species are the growth temperature, the rate of liquefaction of gelatin and the degree of anaerobic growth (in the depths of stab cultures). The first validly-named bacterium recognizable with reasonable certainty as a mesophil is *Bacillus violaceus* Eisenberg 1888; the first recognizable psychrophil is *Bacillus janthinus* Plagge & Proskauer 1887 (syn. *Bacillus lividus* Eisenberg 1891, *Chromobacterium lividum* Holland 1920). Even if further work shows that the two species should be further divided it is essential to decide the valid names and to fix their types by designating cultures.

There seems no cogent reason why the type species should be either the mesophilic or the psychrophilic species, but I have proposed that the type be a mesophil and that it retain the specific epithet 'violaceum' on the following grounds: (1) this epithet was the one most generally used by early authors for mesophilic strains; (2) mesophilic strains appear on the whole to have been the more widely studied, and comprise the majority of the oldest strains available (for instance strains in the American Type Culture Collection (see Table 1)). The valid name for the psychrophilic species would then appear to be *Chromobacterium lividum* (Eisenberg 1891) Holland 1920. The epithet 'lividum' seems to be the earliest which is not illegitimate under Rule 24 (4) (Editorial Board, 1953, p. 52; International Committee, 1953, p. 158).

Type strains have been selected and deposited in the National Collection of Type Cultures and the American Type Culture Collection. The strains already in these collections have features which suggest they show less variation and therefore newer and typical isolates have been chosen. The type strain of the mesophilic species is strain MK (NCTC 9757, ATCC 12472). The type strain of

the psychrophilic species is strain HB (NCTC 9796, ATCC 12473). They have been proposed as neotypes.

A short definition of the genus and the two species under the proposed names is given below.

Genus *Chromobacterium* Bergonzini 1879, p. 38, *emend.* Buchanan 1918,  
p. 52, *nomen conservandum propositum*

Bacteria belonging to the same genus as the proposed neotype species generally have the characteristics given below when tested by the techniques described in this paper. They are rod-shaped Gram-negative non-sporing bacteria which grow on ordinary media and are aerobic; some are also facultatively anaerobic. They are motile by both polar and peritrichate (lateral) flagella, show bipolar or barred staining, and produce a violet pigment (violacein) which is insoluble in water and in chloroform, but is soluble in ethanol, in which solvent it has a maximum optical density near 580 m $\mu$ . and minimum near 430 m $\mu$ .

They show the usual resistance to heat, die rapidly in culture and are not obligate halophils; they utilize citrate for growth but not malonate, and produce acid without gas from glucose. They show the 'catalase' effect, reduce nitrates and destroy nitrites, and they are indole-negative, MR-variable, VP-negative, catalase-positive, NH<sub>3</sub>-positive and urease-negative. The type species is *Chromobacterium violaceum*.

Species 1. *Chromobacterium violaceum* Bergonzini 1880, p. 153, *nomen conservandum propositum*.

Bacteria belonging to the same species as the proposed neotype strain possess the features of the genus and generally also those listed below.

They grow at 37° but not at 4°; the rods in young cultures average about 0.7  $\mu$ . broad and 2  $\mu$ . long, and contain abundant fat. They are markedly proteolytic and haemolytic, give a positive egg-yolk reaction and produce hydrogen cyanide; they utilize citrate slowly; are facultative anaerobes and produce acid from glucose anaerobically. They produce acid in trehalose and do not hydrolyse aesculin.

The proposed neotype strain is strain MK (NCTC 9757, ATCC 12472), whose reactions are described in detail in the tables and text.

Species 2. *Chromobacterium lividum* (Eisenberg 1891, p. 81), Holland 1920, p. 219 (syn. *Bacillus janthinus* Plagge & Proskauer 1887, pp. 463, 464).

Bacteria belonging to the same species as the proposed neotype strain possess the features of the genus and generally also those listed below.

They grow at 4° but not at 37°; the rods in young cultures average about 1.0  $\mu$ . broad and 3.7  $\mu$ . long, and contain little fat. They are feebly proteolytic and feebly haemolytic, do not give a positive egg-yolk reaction and do not produce detectable amounts of hydrogen cyanide. They utilize citrate rapidly, are strictly aerobic and produce acid from glucose but only aerobically.



They do not produce acid from trehalose peptone water but they hydrolyse aesculin.

The type strain is strain HB (NCTC 9796, ATCC 12473) whose reactions are described in detail in the tables and text.

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## The Change from Polar to Peritrichous Flagellation in *Chromobacterium* spp.

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**SUMMARY:** Some strains of *Chromobacterium* exhibit either polar or mixed polar and peritrichous flagellation according to the conditions of culture. Polar and lateral flagella differ in the site of insertion, the wavelength, ease of staining, and in antigenic composition. Peritrichous flagellation is greatest in young cultures on solid media and is least in liquid media. The peritrichate flagella disappear in old agar plate cultures, leaving polar flagellated organisms.

The arrangement of flagella has an important place in modern systems of bacterial classification. A species is usually considered to have either polar or peritrichate flagella but not both, and this feature is one of the main distinctions between, for instance, the pseudomonads and the enterobacteria. It was noticed that one strain of *Chromobacterium* appeared to possess two types of flagellum—a polar flagellum and a lateral flagellum of shorter wavelength. This was misinterpreted (Sneath, Whelan, Singh & Edwards, 1953) until the same appearance was found in the majority of strains of *Chromobacterium* (*sensu stricto*—i.e. the ‘*Bacterium violaceum*’ group). Published descriptions of flagellation in the genus vary considerably, and it is clear that this appearance has been observed before, but without attracting comment. Thus Cruess-Callaghan & Gorman (1933) published photographs similar to Pl. 1, fig. 2, but attributed the variation to species differences; this has not been confirmed (Sneath, 1956). Lehmann & Neumann (1899, **1**, Pl. 23, figs. XI, XII; **2**, p.180) showed drawings of predominantly peritrichate and predominantly polar strains, and commented that some strains showed both forms. Bampton (1913) somewhat ambiguously reported ‘Geisseln 3–4 peritrich, 1–2 polar’. Most other authors report a single polar flagellum. Leifson & Hugh (1953) described certain strains of *Aeromonas* and *Vibrio* spp. which may show either peritrichate or polar flagella. In *Aeromonas* sp. the organisms in early phases of growth showed peritrichate flagella, and in old cultures only polar flagella.

### METHODS

**Strains.** The strains are those described previously (Sneath, 1956), all belonging to the ‘*Bacterium violaceum*’ group.

**Flagella staining.** The organisms were stained by the method of Leifson (1951). Cultures on solid media were suspended in distilled water, care being taken to avoid the picking up of fragments of medium. Liquid cultures were washed three times by centrifugation with distilled water before staining.

**Media.** Nutrient broth was that of Hartley (1922), and nutrient agar was made by adding to it 1.5% (w/v) Japanese agar. Blood agar was nutrient

agar containing 5% (v/v) sterile defibrinated horse blood. The glucose ammonia medium was that of Mandelstam (1954) with omission of sodium lactate.

*Incubation.* For mesophilic strains the incubation temperature used routinely was 30°, and for psychrophilic strains 25°.

*Antisera and agglutination tests.* Antisera were prepared by injecting washed organisms (suspended in 0.04% formaldehyde in physiological saline) into rabbits intravenously three times a week, increasing the dose gradually from about  $10^8$  to  $10^9$  organisms. Seven injections were given. The suspension of strain DK with only polar flagella (referred to subsequently as polar suspension) was a nutrient broth culture grown for 24 hr. at 35°. The suspension of the same strain with both polar and lateral flagella (peritrichate suspension) was the growth from a blood agar culture of strain DK grown at 30° for 18 hr. Suspensions prepared in the same way, and similarly formalized, were used for absorption and agglutination, and were all stained to confirm that they were in the required phase. Deflagellated organisms were prepared by treating living cultures in a Waring Blendor until there were very few motile organisms, and few flagellated organisms in stained preparations; the time required was usually about 6 min. Agglutination tests were performed in 0.17% sodium chloride: 0.25 ml. amounts of diluted antiserum and of suspension (of an opacity of 5.5 International Opacity Units (World Health Organization, 1954)) were mixed in Dreyer's tubes and incubated at 56°. The titre given is the reciprocal of the final dilution of antiserum in the last tube with agglutination just visible to the naked eye.

## RESULTS

Two types of organism were found: those with only a single polar flagellum (Pl. 1, fig. 1), and those with lateral flagella in addition to a single polar flagellum (Pl. 1, fig. 2). For brevity the former are referred to as polar organisms and the latter as peritrichate organisms. It was uncommon to see organisms with lateral flagella which lacked a polar flagellum, or to see organisms with two polar flagella (unless they were dividing, and they were then always at opposite poles).

All strains were examined after 18 hr. growth on nutrient agar plates, and thirty out of thirty-eight strains showed both polar and lateral flagella (Sneath, 1956). Some strains were studied extensively, and these can be roughly divided into two groups: (1) those showing only polar flagellation (Pl. 1, fig. 1) under all conditions examined (strains FH, MW, AM and MH), and (2) those showing during early growth on agar many lateral flagella, usually 4–6, of great length and of very short wavelength (Pl. 1, fig. 2), in addition to the polar flagellum (strains LG, LW, BN, BH, RT, DK, SH, NT, RV and MK). With all these strains the number of lateral flagella decreased as the plate cultures became aged, and usually only polar flagellated organisms were found after 2 days of incubation. The two classes were not sharply defined, and some strains, e.g. BH, appeared intermediate (with only a few lateral flagella). The classes did not appear to be related to other characteristics of the strains, such as pathogenicity or biochemical reactions. All attempts

to obtain stable variants in one or other phase have proved unsuccessful, but the existence of strains possessing only polar flagella suggests that these may be variants which have lost the ability to produce lateral flagella.

#### *Differences between polar and lateral flagella*

The differences between the two types of flagellum, as seen in stained preparations, is generally quite clear. Although there is some variation from strain to strain, most strains possess a short polar flagellum, 2–4  $\mu$ . long, with a wavelength *c.* 2  $\mu$ . and amplitude *c.* 0.35  $\mu$ . and lateral flagella 3–10  $\mu$ . long, with wavelength *c.* 1.25  $\mu$ . and amplitude *c.* 0.4  $\mu$ . The lateral flagella stain easily, but except in some strains such as AM and MH, the polar flagellum stains more faintly. These two strains also have polar flagella which are longer than usual (4–6  $\mu$ .) though the wavelength is about the same.

The polar flagellum has only been observed arising from the extreme tip of the cell. The lateral ones may show subpolar or lateral attachment.

Flagellar staining is much beset by artifact formation, and therefore some electron micrographs were made for comparison. The same morphological features are seen as in stained preparations (Pl. 1, figs. 3–6). As with stained preparations, previous fixation in 0.4% formaldehyde before drying and staining did not alter the shape of the flagella. In lysed organisms a number of basal granules were visible, from which both forms of flagella appeared to originate; these granules are also visible in Pl. 1, fig. 6, which is a cast of the bacterial surface. Houwink & van Itersen (1950) found in a bacillus isolated by Gray & Smith (1950) that the polar flagella were thicker than the lateral ones. No difference in thickness was seen in *Chromobacterium* spp.

It appears unlikely that the flagellar changes are due to multicellularity of the rods during early growth (each cell having one flagellum), since nuclear stains by the acid hydrolysis Giemsa method (Murray, Gillen & Heagy, 1950) and cell-wall stains by the method of Webb (1954) did not show more numerous nuclei or cross-walls in the peritrichate organisms. Both polar and peritrichate organisms usually showed two or four nuclei, and usually no cross-walls.

#### *Antigenic difference*

It had been noted that some rabbit antisera gave only partial agglutination of some strains over a wide range of serum dilutions, as if the suspension contained two antigenic types. This suggested that the antigens of the polar and lateral flagella were different, so that 'polar' suspensions would possess only the polar H antigen, while 'peritrichate' suspensions would possess both H antigens. Antisera were therefore made by injecting polar and peritrichate suspensions of strain DK, and a mirror absorption test was performed. The results are shown in Table 1; it will be seen that absorption with polar flagellate organisms leaves an antibody fraction ('anti-lateral' antibody) which agglutinates only the peritrichate organisms, and this suggests that the peritrichate organisms contain an extra antigen. It is also clear that the polar suspension



used for immunization contained some of this additional antigen, which has produced a low titre of antibody.

It seemed possible that the antigens of the polar and peritrichous flagella might be the same, and that the results in Table 1 might be due solely to the smaller amount of flagellar substance on polar-flagellated organisms (which could be as little as 1/20 of that on peritrichate organisms). This is rendered very unlikely by the following experiment. The antiserum against peritrichate

Table 1. *Cross-absorption of antisera by suspensions of polar or peritrichate organisms of Chromobacterium sp. strain DK*

Serum was absorbed by mixing with an equal volume of a 20 % (v/v) suspension of packed organisms, and centrifuging after 1 hr. at 35°. The titrations were incubated at 56° for 4 hr.

Antiserum to	Absorbed with	Titre against	
		Polar organisms	Peritrichate organisms
Polar organisms	Unabsorbed	640	1,280
Polar organisms	Polar organisms	20	640
Polar organisms	Peritrichate organisms	40	20
Peritrichate organisms	Unabsorbed	320	10,240
Peritrichate organisms	Polar organisms	40	5,120*
Peritrichate organisms	Peritrichate organisms	40	1,280†

\* The titre was not significantly decreased by two further absorptions with polar organisms.

† The titre was decreased to 80 by a second absorption with peritrichate organisms.

Table 2. *Fractional absorption of antiserum to peritrichate organisms of Chromobacterium sp. strain DK*

Samples (2 ml.) of a dilution of antiserum were mixed with 50 % (v/v) packed organism suspensions. Saline was added to make 4 ml., and the tubes centrifuged after 1 hr. at 35°. The absorbed sera were titrated against peritrichate organisms (the serum was too dilute to agglutinate polar organisms) and incubated for 4 hr. at 56°.

Absorbing suspension	Amount of absorbing suspension added (ml.)	Titre against peritrichate organisms
Unabsorbed	0	32
Peritrichate	0.002	8
Peritrichate	0.02	2
Peritrichate	0.4	0
Polar	0.4	16

organisms was diluted so as to give a final titre of 32 with peritrichate organisms, and was absorbed with various amounts of polar and of peritrichate organisms. It is seen (Table 2) that the polar organisms have less absorbing power than 1/200 of the same amount of peritrichate organisms. These results might also be due to the production of an extra O antigen under the conditions causing peritrichate flagellation. This is an unlikely explanation, since peritrichate organisms which had been deflagellated in a Waring Blendor were inagglutinable by the absorbed 'anti-lateral' serum. Also the agglutination of peritrichate organisms was typical flocculent 'H' agglutination, appearing within 2 hr. Similar purification of an 'anti-lateral' H antibody was also obtained

with strain BN, by absorbing an anti-BN 'OH' antiserum with polar-flagellated organisms of that strain.

Positive proof of the existence of a 'polar' antigen was obtained by incubating agglutination tests for 18 hr. at 56°. It was noted by Gardner & Venkatraman (1935) that long incubation at high temperatures was needed to produce H agglutination of the monotrichate cholera vibrio, evidently because monotrichate organisms form large agglutinates with difficulty. A sample of antiserum against polar-flagellated organisms of strain DK was absorbed with an equal volume of a 50% suspension of polar organisms which had been deflagellated in a Waring Blendor, and the absorbed serum was titrated against deflagellated polar and peritrichate suspensions (incubated 18 hr. 56°). The results (Table 3) show that an antibody fraction remains which agglutinates polar suspensions but not deflagellated suspensions. This fraction was removed when the absorbed serum was absorbed with polar organisms. Similar results were obtained when living suspensions were used for absorption and agglutination. The agglutination was a fine, flaky type not unlike O agglutination, and did not appear after incubation for 4 hr. at 56° followed by standing at 20° overnight.

Table 3. *Absorption of antiserum against polar flagellated organisms of Chromobacterium sp. strain DK to demonstrate polar 'H' agglutination*

	Titre (56°, 18 hr.) against		
	Deflagellated organisms	Polar organisms	Peritrichate organisms
Unabsorbed serum	256	512	512
Serum absorbed with deflagellated organisms	8	128*	256

\* The titre was decreased to 64 by a second absorption with deflagellated organisms, but was decreased to 8 by an absorption with polar organisms.

The antigen of the polar flagella is thus evidently distinct from that of the lateral flagella, since the lateral H antibody does not agglutinate polar organisms (a sample absorbed twice with polar organisms had, at 56° for 18 hr., a titre of 2048 with peritrichate organisms, but a titre of only 2 with polar and with deflagellated organisms), nor do polar organisms absorb this antibody. The polar H antiserum contained lateral H antibody (see Tables 1 and 3); it was not possible to remove this by further absorption, since peritrichate organisms also have polar flagella, and both antibodies were removed by them. No variants lacking the polar flagellum have so far been found and therefore pure polar H antisera cannot yet be prepared by absorption with peritrichate organisms. As seen in Table 1, polar suspensions contain enough lateral flagella, a good antigen in rabbits, to induce formation of lateral H antibody.

#### *Effect of cultural conditions upon the type of flagellation*

The strains DK and LG were studied intensively in an attempt to discover the cause of the flagellar changes. This has not been found, but it was observed that lateral flagella were abundant (3-6/organism) in young nutrient agar plate

cultures, and were most abundant (5–10/organism) in blood agar cultures. Lateral flagella were rare or absent on organisms grown in liquid media. Changes in the following factors did not alter the form of flagellation to any great extent in liquid media: anaerobiosis, aeration, growth temperature, age of culture, growth-pH value, concentration of glucose, amino acids (casein hydrolysate), ammonium, sodium, cyanide, potassium, chloride, phosphate, sulphate, magnesium or calcium ions. Addition of granules of agar to broth or to glucose ammonia medium caused the appearance of a few lateral flagella only. Addition of fresh broth to 18 hr. broth cultures did not cause the appearance of lateral flagella. Charcoal had no effect. Growth in liquid exuded from nutrient agar slopes showed mainly polar forms. Lateral flagella were abundant (3–6/organism) in young cultures on silica-gel nutrient broth plates, and cultures upon cellophan floating on nutrient broth also showed many lateral flagella (2–3/organism). Various trace elements were tested without positive results. Attempts to relate the flagellation to the effective viscosity of the medium (which is presumably high within colonies on solid media) were inconclusive.

#### DISCUSSION

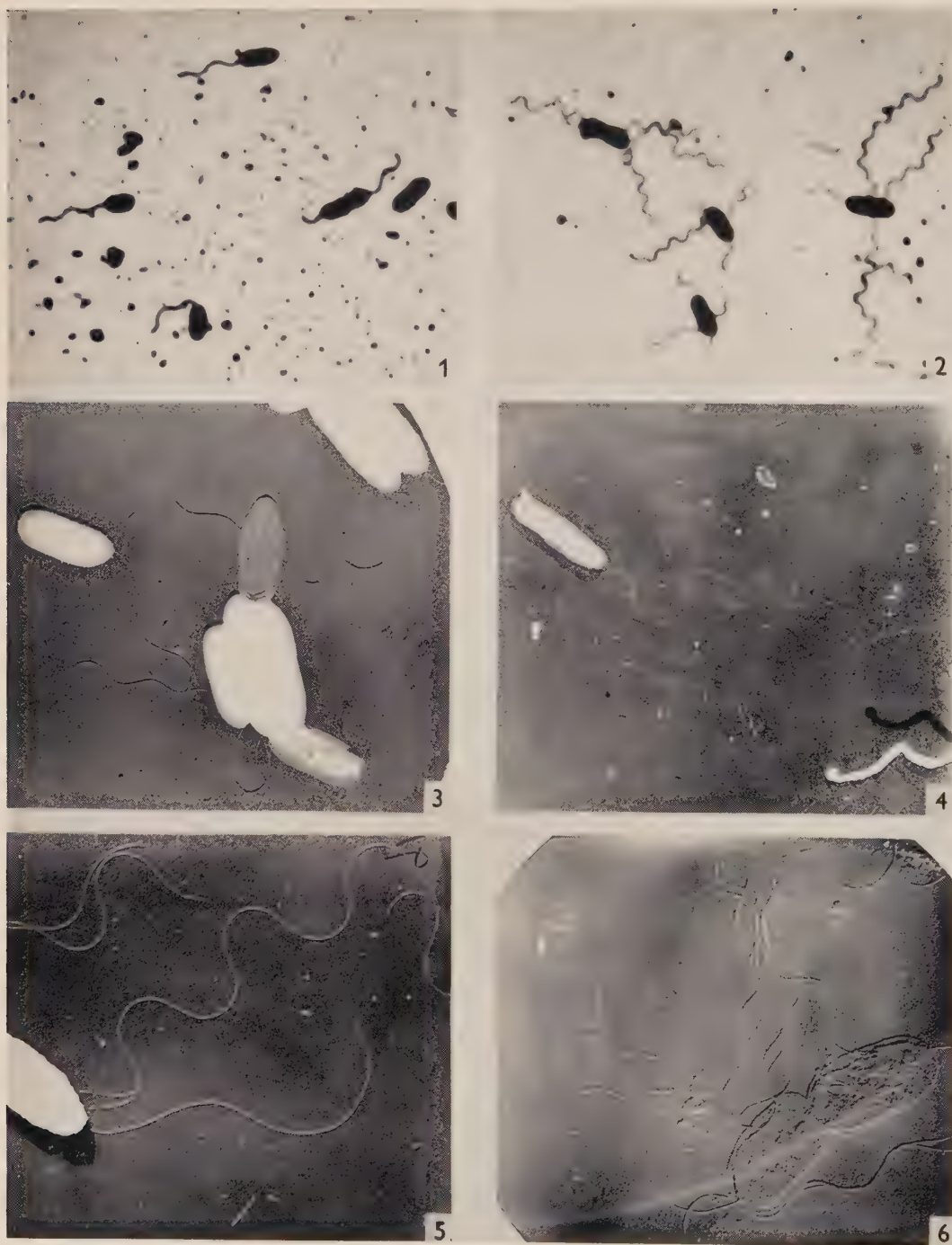
The appearance of lateral flagella seems to be caused by the environment, and not by the selection of variants, since peritrichate organisms can appear upon a plate in large numbers within 4 hr. The variation confirms the observations of Leifson & Hugh (1953) that some bacteria can possess both polar and lateral flagella—with different antigens in one of their instances. It also raises a question of taxonomic importance, for in *Chromobacterium* the peculiar flagellation is the rule rather than the exception. It is clear that the form of flagellation, like any other single characteristic, should not be given undue weight in classification. There are instances in which organisms otherwise identical have been placed in separate genera on account of a difference in flagellation (as has happened in *Chromobacterium*; e.g. Chester (1901) placed *Bacillus violaceus Laurentius* both in *Bacillus* and in *Pseudomonas* because of differing opinions on whether it had peritrichate or polar flagella). Such differences may well be due to unrecognized changes in flagellation caused by the cultural conditions. These observations also indicate the need for standardization of the conditions of growth if the arrangement of flagella is used in classification.

The phenomenon is also of interest in relation to antigenic phase variation in *Salmonella*. It seems to be assumed that both phases are peritrichate, but I do not know of a study on this point. Three strains of *Salmonella* (one each of *S. typhimurium*, *S. cholerae-suis* and *S. paratyphi* B) were examined by flagellar staining. In each strain both phases were peritrichate and there was no obvious difference in the flagellar morphology.

The author is indebted to Mr R. Valentine for the electron micrographs.







P. H. A. SNEATH—FLAGELLA OF *CHROMOBACTERIUM* SPP. PLATE 1

(Facing p. 105)

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## EXPLANATION OF PLATE

## PLATE 1

*Chromobacterium* spp.; the flagellation, etc., of various strains.

- Fig. 1. Strain MH, from 24 hr. nutrient agar plate culture. Flagella stain,  $\times 2250$ . Polar flagellated organisms.
- Fig. 2. Strain SH, from 24 hr. nutrient agar plate culture. Flagella stain,  $\times 2250$ . Peritrichate organisms with both polar and lateral flagella.
- Fig. 3. Strain DK, from 24 hr. nutrient broth culture. Electron micrograph, fixed with  $\text{OsO}_4$  vapour, platinum shadowed,  $\times 7700$ . Polar flagellated organisms, and one flagellum of the lateral type.
- Fig. 4. Strain DK, from 8 hr. nutrient agar plate culture. Electron micrograph, fixed with formalin, carbon shadowed,  $\times 8300$ . An organism with one polar and two lateral flagella of subpolar origin.
- Fig. 5. Strain DK, from 18 hr. blood agar plate culture. Electron micrograph, fixed with  $\text{OsO}_4$  vapour, carbon shadowed,  $\times 16,760$ . Polar and lateral flagella.
- Fig. 6. Strain DK from 8 hr. blood agar plate culture, fixed with formaldehyde. Electron micrograph of silicon monoxide replica, platinum shadowed,  $\times 12,000$ . The basal granules of the flagella are faintly shown.

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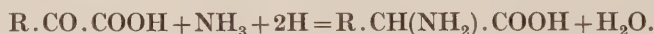
## Studies in Amino Acid Biogenesis: the Synthesis of Alanine from Pyruvate and Ammonia

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**SUMMARY:** Washed suspensions of certain bacteria can synthesize alanine from pyruvate and ammonia. This has been studied in detail in *Bacillus subtilis*. Several other amino acids can be formed from the corresponding keto-acids, but synthesis was most rapid in the case of alanine. Notably, glutamic acid is formed from keto-glutarate relatively slowly, whilst oxalacetate fails to give aspartate. Pyruvate can accept an amino group from a number of amino acids by transamination (but aspartic and glutamic acids are less effective than ammonia itself). It is possible that the reductive amination of pyruvate may proceed directly rather than through transamination from some other primary amino-acceptor.

The last step in the synthesis of an amino acid is often the exchange of the carbonyl group of the appropriate keto acid for an amino group. When the ultimate source of the latter is ammonia we can refer to the reaction as 'reductive amination':



This reaction has been most studied in the case of pyruvic acid and alanine. It was first demonstrated in animals by Embden & Schmidt (1912) but was not investigated in detail until Kritzman (1947) examined the reaction in liver and kidney slices. Since then it has been demonstrated in washed suspensions of *Clostridium* sp. (Nisman, Rayneaud & Cohen, 1947) and in *Aerobacter aerogenes* (Paretsky, 1948). Holden, Wildman & Snell (1951) have also shown that *Lactobacillus* will grow on media in which certain essential amino acids have been replaced by the corresponding keto acids. Extracellular production of alanine has been demonstrated in *Corynebacterium diphtheriae* growing on casein hydrolysate medium (Linggood & Woiwod, 1949), while Goodlow, Mika & Braun (1950), Braun, Goodlow, Kraft, Altenbern & Mead (1951), and Altenbern & Housewright (1951) showed that extracellular alanine production in *Brucella abortus* had an interesting significance as a determining factor in the rough-smooth transformation. Most of their work was concerned with the pyruvate-asparagine transamination, but they also showed that alanine could be slowly formed from ammonia and pyruvate.

The first suggestion as to the mechanism of the reaction came from Kritzman's (1947) work with liver slices (cf. Kritzman & Melik-Sarkisyan, 1948):

- (1) Pyruvate +  $CO_2$  = oxalacetate,
- (2) Oxalacetate +  $NH_3$  = aspartate,
- (3) Aspartate + pyruvate = oxalacetate + alanine.

He supported this scheme by showing that carbon dioxide or one of the dicarboxylic acids was required and that (in the presence of carbon dioxide),

aspartate appeared diminished and alanine was the sole final product. Nisman *et al.* (1947) found an initial production of aspartate and glutamate when using washed suspensions of *Clostridium*, and Konikova, Kritzman & Jakobson (1948) and Konikova, Kritzman, Jakobson & Samarina (1949) reported similar results with *Escherichia coli*. (See also Kritzman, Jakobson & Konikova (1948); Vishepan & Mogilevsky (1948); and Jakobson, Konikova, Kritzman & Melik-Sarkisyan (1949). In most cases only summaries of the Russian papers have been available to us.) Paretsky (1948), however, found no carbon dioxide requirement for formation of alanine from pyruvate and ammonia in cell-free extracts of *Aerobacter aerogenes*.

The object of the present work was to investigate the characteristics of the system and the mechanism of the reaction in *Bacillus subtilis*.

### METHODS

A strain of *Bacillus subtilis* (strain 4 in our list) was used throughout except for the comparative experiments cited in Table 8. The medium used was Nutrient Broth No. 1 (Oxo, Ltd., London), supplied in the form of dry granules. It contained Lab-Lemco, 0.1%; yeast extract, 0.2%; peptone, 0.5%; NaCl, 0.5%. Towards the end of this work we found that Nutrient Broth No. 2 (Oxo, Ltd.; Lab-Lemco, 1.0%; peptone, 1.0%; NaCl, 0.5%) gave more active suspensions and this medium was used for the experiments in Table 8.

*Washed suspensions.* Roux bottles containing 150 ml. medium were inoculated with a few drops of a young broth culture and incubated at 37° for 15–17 hr., i.e. until the culture was near the end of the logarithmic phase of growth. This gave maximum enzyme yield though earlier harvesting would have given greater specific activity. Old cultures were less active and gave much greater blanks for amino-N in the absence of substrate. The organisms were collected by centrifugation in glass tubes. When plastic tubes were used the suspensions were almost inactive; the reason for this is not known. After washing twice on the centrifuge with distilled water the organisms were suspended in a suitable volume of 0.2M phosphate buffer pH 7.0, to give a concentration of 15–30 mg. dry wt./ml. Repeated washing in water or saline did not impair activity (cf. Konikova *et al.* 1948). The density of the suspensions was determined turbidimetrically using the EEL photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex), which was calibrated by dry-weight determinations. In the experiment on age-of-culture effect (Fig. 4) each suspension was standardized by dry-weight determination to avoid errors arising from variation of the turbidity factor with age. Suspensions suffered no appreciable loss in activity on storage at 0° for up to 48 hr.

*Reagents.* During the earlier stages of this work sodium and ammonium pyruvate were prepared in solution as required, by neutralizing redistilled pyruvic acid with sodium or ammonium hydroxide at 0°. Later commercial sodium pyruvate became available and pyruvic acid was no longer used; ‘ammonium pyruvate’ was obtained by using a solution of sodium pyruvate and ammonium sulphate. Phenylpyruvic acid was prepared according to

Herbst & Shemin (1939). We gratefully acknowledge gifts of sodium  $\alpha$ -keto-butyrate and  $\alpha$ -ketoisovalerate from Dr C. Long (Aberdeen); sodium fluoracetate from Dr B. D. Saunders (Cambridge); sodium  $\alpha$ -ketoisocaproate from our colleague Dr W. Lijinsky.

*Standard experimental technique.* All washed suspension experiments—including determinations of  $Q_{\text{Alanine}}$ —were performed under the following standard conditions, except where otherwise stated. The buffered bacterial suspension (40–60 mg. dry wt. of organisms), the substrate (ammonium pyruvate, final concentration 0.12M), and any special reagents (inhibitors, etc.) were placed in a 15 ml. conical glass centrifuge tube, making a total volume of 6 ml. with a final strength of buffer (phosphate, pH 7) 0.1M. The tube was placed in a 37° water bath for 3 hr. Occasionally the organisms tended to settle on the bottom of the tube and periodic mixing was necessary. The reaction was stopped by addition of 0.3 ml. 2N-HCl. In a few experiments (referred to below) the reaction was stopped by addition of trichloroacetic acid, and in the transamination experiments by heating to 100° for 5 min. This avoided the introduction of unwanted anions which might have interfered with the subsequent ion-exchange treatment. In all cases the organisms were centrifuged and discarded and the supernatant fluid stored at 0° till required. Blanks were performed by incubating substrate and suspension separately and mixing only at the end of the incubation period. The blank amino-N was usually negligible (Table 1).

*Paper chromatography.* Orthodox techniques were used; descending single-dimensional chromatograms were run for 24–48 hr. with a solvent mixture consisting of butanol (480 ml.), acetic acid (95 ml.), butyl acetate (35 ml.) and water (195 ml.). This system readily resolved alanine from all other amino acids normally found in proteins. In some cases results were confirmed by using the phenol + water system.

*Determination of amino acids.* In most experiments paper chromatography showed that only one amino acid was present (usually alanine). This amino acid could then be estimated by the nitrous acid method of Van Slyke (1929). Ammonia was present in most experiments and interfered with this method. It was removed by adding NaOH till the solution was alkaline to thymol blue, and boiling gently for a further 0.5 min. after the smell of ammonia had disappeared. Control runs showed that this removed the ammonia without loss of amino acid. In the transamination experiments where aspartic acid or glutamic acid were present as well as alanine, the latter was separated by an ion-exchange method described below.

*Results* are expressed in most cases in terms of  $Q_{\text{Alanine}}$ , defined as  $\mu\text{l.}$  of alanine produced per mg. (dry wt.) of organism per hour. Densities of suspensions of *Bacillus subtilis* 4 were determined turbidimetrically, the instrument being calibrated by dry-weight determinations; but the results in Table 8 are based on nitrogen determinations and are expressed as  $q_{\text{Alanine}}^{\text{N}}$ .



## RESULTS

*Characteristics of the system*

*Variance between replicates.* Washed suspensions were prepared from 18 identical cultures of *Bacillus subtilis* 4 and the  $Q_{\text{Alanine}}$  value for each was determined under standard conditions. The coefficient of variation of an individual determination was 5.2%.

Table 1. *Synthesis of alanine*

Reagents and suspension were incubated together at 37°, the reaction stopped by addition of 0.06 ml. of 2N-HCl, the organisms removed by centrifugation and the amino-N determined in the supernatant.

<i>B. subtilis</i> (50 mg./ml.) (ml.)	2	2	2	2
Sodium pyruvate (0.5M) (ml.)	3	3	3	—
Ammonium sulphate (0.25M) (ml.)	3	3	—	3
Water (ml.)	—	—	3	3
Incubation time (hr.)	3	0	3	3
Alanine produced (mg. N)	5.02	0.05	0.00	0.08

*Overall reaction.* Table 1 shows that pyruvate and ammonia were required. In this experiment the concentrations of both organism and substrate used were a little higher than in the standard technique, to facilitate detection of any alanine formed in the absence of substrate or suspension. Although the reaction involves reduction no separate reducing agent was required, the pyruvate itself acting as hydrogen donor and the addition of a separate H-donor, e.g. formate did not accelerate the reaction. Up to 25% of the pyruvate metabolized was converted to alanine; the remainder underwent dismutation. The fate of the pyruvate was discussed in a preliminary note by Sewell & King (1954). The ammonia is used exclusively for alanine formation (Table 2).

Table 2. *Nitrogen balance in alanine synthesis*

A suspension of *Bacillus subtilis* (4 ml., 100 mg. total dry wt.) was incubated with ammonium sulphate (1 ml. of 0.25M) and sodium pyruvate (6 ml. of 0.5M), for 3 hr. at 37°. 3 ml. of 10% trichloroacetic acid was added and the material centrifuged. Total N was determined in the deposit. The supernatant was assayed for ammonia and alanine. In the control, trichloroacetic acid was added *before* incubation.

	Expt. (mg. N)	Control (mg. N)
'Protein'	5.88	5.86
Alanine	3.89	0.00
Ammonia	3.15	7.17
Total	12.92	13.03

*Product of reaction.* Alanine was demonstrated by paper chromatography and by isolation and chemical characterization; it proved to be the DL-mixture (Fairhurst, 1952).

*Time of incubation.* A 3 hr. incubation period at 37° was normally used; alanine synthesis was almost linear over this period but further incubation produced an appreciable falling-off in the rate of synthesis.

*pH value of optimum activity.* The fairly sharp optimum at pH 6.8–7.0 (Fig. 1) was lower than the figure of pH 8.5 given by Kritzman *et al.* (1948) for liver.

*Concentration of  $\text{NH}_4^+$ .* The  $Q_{\text{Alanine}}$  value was determined in a series of experiments in which the ammonium sulphate concentration was varied from 0.5 to 0.03 M. The pyruvate concentration was constant at 0.25 M. Thin suspensions (2 mg./ml.) were used to ensure that, even at the lowest  $\text{NH}_4^+$  concentration, 80% of the ammonia would remain unchanged. Otherwise, the experiments were performed under standard conditions. Fig. 2 shows that even the lowest ammonium sulphate concentration (0.03 M) saturated the system.

*Concentration of pyruvate.*  $Q_{\text{Alanine}}$  was determined in a series of experiments in which the pyruvate concentration was varied. The ammonium sulphate concentration was constant at 0.12 M. Again thin suspensions were used, and incubation also was shortened to 2 hr. This ensured that the pyruvate concentration did not fall significantly during the experiment even at the lowest concentrations used (0.015 M). Pyruvate determinations were performed at the end of the experiment and showed that in no case had more than one-third of the pyruvate been utilized. The Michaelis constant,  $K_s$ , was determined by the reciprocal plot method of Lineweaver & Burk (1934); Fig. 3 gives the plots for experiments with two different suspensions, giving  $K_s$  values of 0.12 and 0.13 M respectively. It is not known, however, whether these surprisingly high values represent a true affinity constant for the system or whether other factors are involved such as competition for pyruvate by other metabolic pathways.

*Presence of oxygen.* The majority of experiments were performed in test tubes with no special precautions to ensure either aeration or anaerobiosis. No increased activity was found when nitrogen-filled Thunberg tubes were used, but vigorous aeration had a marked adverse effect. This accords with the findings of Altenbern & Housewright (1951) for alanine synthesis in *Brucella abortus*; see also Fowler & Werkman (1955). We found the alanine yield decreased by about half on shaking in air-filled Warburg manometers; more thorough aeration reduced the yield to zero; activity was fully restored when aeration was stopped. Whether this was a true inhibition of the enzyme or whether it arose from the diversion of the pyruvate to other metabolic channels is not known. The organism itself cannot grow under strict anaerobiosis.

*Nature of keto acid.* Various keto acids (neutralized to pH 7 with NaOH) were incubated with ammonium sulphate under standard conditions, save that the final concentration of keto-acid was 0.25 M, and the reaction was arrested by addition of trichloroacetic acid (final concentration, 2.5%). Parallel experiments with pyruvate were set up in each case. The cells were removed by centrifugation and the supernatants examined by paper chromatography and assayed for amino-N. In each case only one amino acid was found. The relative rates of formation were (alanine=100), valine from  $\alpha$ -keto-*iso*-valeric acid, 65; leucine from  $\alpha$ -keto-*iso*-caproic acid, 50;  $\alpha$ -aminobutyric acid from  $\alpha$ -ketobutyric acid, 40; glutamic acid from  $\alpha$ -ketoglutaric acid, 10. No reductive amination of phenylpyruvic acid was observed. Oxalacetic acid gave

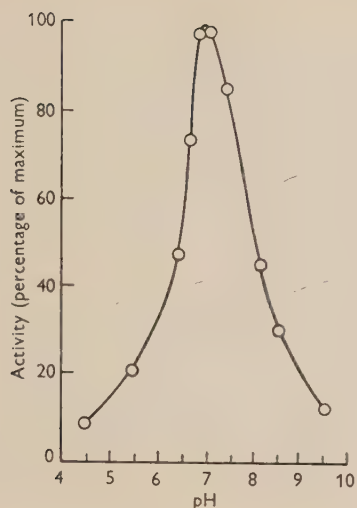


Fig. 1.

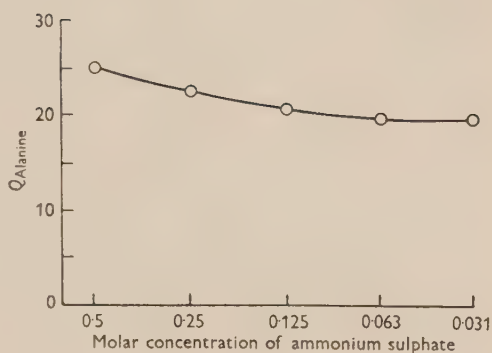


Fig. 2.

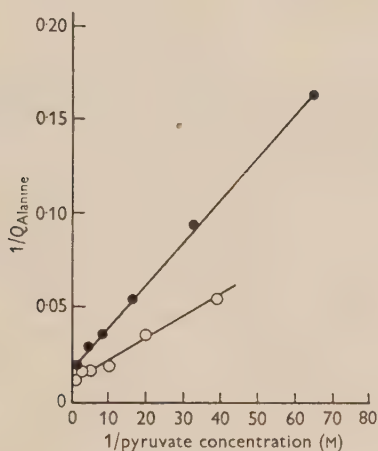


Fig. 3.

Fig. 1. Effect of pH on rate of alanine synthesis.  $Q_{\text{Alanine}}$  was determined under standard conditions, the reagents being buffered with 0.1 M-acetate (pH 4.5–5.5), 0.1 M-phosphate (pH 6–8) or 0.1 M-borate (pH 8.5–9.5).

Fig. 2. Relation between ammonium salt concentration and rate of alanine synthesis.  $Q_{\text{Alanine}}$  was determined under standard conditions save that the final concentration of the organism was 2 mg./ml. dry wt. Sodium pyruvate concentration, 0.12 M; final concentrations of ammonium sulphate indicated on abscissa.

Fig. 3. Relation between pyruvate concentration and rate of alanine synthesis: reciprocal plots of two experiments. (Method of Lineweaver & Burk, 1934.)  $Q_{\text{Alanine}}$  was determined under standard conditions, except that thin suspensions were used (final concentration of organism, 1 mg./ml. (upper line) and 0.5 mg./ml. (lower line) and the incubation time was reduced to 2 hr. This ensured that the pyruvate concentration was not significantly reduced during the experiment, even at the lowest concentration employed. Pyruvate estimations at the end of the experiment confirmed this. Ammonium sulphate concentration, 0.12 M throughout. Reciprocal of  $Q_{\text{Alanine}}$  ( $1/V$ ) plotted against reciprocal of molar pyruvate concentration ( $1/S$ ).



considerable amounts of alanine but no aspartic acid; presumably it was decarboxylated to pyruvate, which then formed alanine. The negative results with oxalacetic acid and the slow reaction of  $\alpha$ -ketoglutaric acid were important as they might be regarded as excluding these substances as intermediates in the formation of alanine. Two possibilities must be considered: (1) Failure of the keto acids to penetrate the cell membrane. This was investigated by freeing a suspension of the organism from endogenous substrates (as far as possible) by aerating for  $1\frac{1}{2}$  hr. at  $37^\circ$ , and examining its capacity for dehydrogenating various substrates on incubation with methylene blue in Thunberg tubes (Table 3). Since both oxalacetate and  $\alpha$ -ketoglutarate were more rapidly oxidized than formate or pyruvate, it seems reasonable to suggest that they could enter the cell readily. (2) It had been assumed that oxalacetate and  $\alpha$ -ketoglutarate (like pyruvate) need no separate H-donor for reductive amination, and their ready reduction of methylene blue would support this view. However, suspensions of the organism were incubated with the keto acids and ammonia in the presence of formate, shown to be an active H-donor (Table 3); no increase in reductive amination was observed.

Table 3. *Reduction of methylene-blue by various substrates in the presence of organism*

The body of each Thunberg tube contained 1 ml. substrate (0.1 M), 2 ml. of 0.2 M-phosphate buffer, pH 7, and 1 ml. of 0.0002 % methylene blue. The hollow stoppers contained 1 ml. (9.5 mg. dry wt.) of washed suspension of *Bacillus subtilis*. The tubes were filled with oxygen-free nitrogen and placed in a water bath at  $37^\circ$ ; the contents were mixed after 12 min. and the time required for complete reduction recorded.

Substrate	Reduction time (min.)
None	7.5
Sodium pyruvate	2.0
Sodium $\alpha$ -ketoglutarate	1.1
Sodium oxalacetate	1.5
Sodium formate	2.5

*Search for intermediate products.* Washed suspensions were incubated with pyruvate and ammonia under standard conditions and the products examined by paper chromatography at 20 min. intervals up to 2 hr. A steady production of alanine was found, without trace of any other amino acid.

*Carbon dioxide not required.* The formation of carbon dioxide by dismutation of pyruvate (Sewell, 1954) makes establishment of 'CO<sub>2</sub>-free' conditions difficult. An experiment (Table 4) was performed, however, in which we endeavoured to remove the carbon dioxide as fast as it was formed by working at pH 6.5 and shaking in Warburg manometers with KOH in the centre cups. At pH 6.5 reductive amination is still fairly rapid but carbon dioxide is readily expelled from the solution. Table 4 shows that the 'CO<sub>2</sub>-free' system (Expt. 2) was as active as the control (Expt. 1) in which KOH was not present in the manometer. Addition of oxalacetate or  $\alpha$ -ketoglutarate did not accelerate the reaction (Expts. 3 and 4).

*Transamination.* If the reaction proceeds through a transamination mechanism involving the dicarboxylic acids these should be at least as effective  $\text{NH}_2$ -donors as ammonia itself. This was investigated experimentally. The organism was incubated with the reagents shown in Table 5. The reaction was stopped by heating ( $100^\circ/5$  min.) and the cells removed by centrifugation. The supernatant was passed down a column ( $18 \times 1.2$  cm.) of the cation-exchanger Zeokarb 225 (Permutit Co., London), treated with HCl and washed. This retained the amino acids but passed through pyruvic acid and other anions. The

Table 4. *Effect of carbon dioxide and dicarboxylic acids*

Suspension previously shaken in air for  $1\frac{1}{2}$  hr./ $37^\circ$  to oxidize endogenous metabolites. All reagents (except KOH) buffered at pH 6.5 with 0.1 M-phosphate. Manometers filled with oxygen-free nitrogen and shaken for 1 hr./ $37^\circ$  before tipping. Shaking continued for further 3 hr. Manometers dismantled and pH determined; no change detected. 0.9 ml. 10% trichloroacetic acid added. Organisms spun down and supernatants assayed for amino-N. Controls were identical with experimental series but side-bulb tipped at end of experiment.

	Expt. ...	1	2	3	4
Main flask	Suspension (40 mg. dry wt.) (ml.)	2	2	2	2
	Buffer, pH 6.5 (ml.)	0.4	0.1	—	—
Centre cup	KOH, 10% (ml.)	—	0.3	0.3	0.3
Side bulb	Ammonium pyruvate, 0.1 M (ml.)	0.6	0.6	0.6	0.6
	Oxalacetate, 0.05 M (ml.)	—	—	0.1	—
	$\alpha$ -ketoglutarate, 0.05 M (ml.)	—	—	—	0.1
	$Q_{\text{Alanine}}$	20.6	20.6	17.0	18.3

Table 5. *Formation of alanine by transamination*

Final concentrations of reagents are given; total volume, 30 ml. in each experiment. Organism and reagents (buffered to pH 7), incubated 3 hr. at  $37^\circ$ . Reaction stopped by heating to  $100^\circ$  for 5 min. Alanine separated and determined as described in text.

Organism (50 mg.)	+	+	+	+
Pyruvate, 0.25 M	+	+	+	+
Ammonium sulphate, 0.025 M	—	+	—	—
Glutamate, 0.05 M	—	—	+	—
Aspartate, 0.05 M	—	—	—	+
Alanine formed (mg. N)	0.12	2.88	0.66	0.60

column was washed with water and then eluted by perfusion with 0.5N-ammonia. The effluent was collected by means of a fraction-collector and the fractions giving a positive ninhydrin reaction were pooled. No separation of the amino acids was attempted at this stage. Most of the surplus ammonia was removed by boiling; the solution was then adjusted to pH 4 (HCl) and passed down a column ( $18 \times 1.2$  cm.) of the anion-exchanger Deacidite FF (Permutit Co., London), previously treated with 0.2 M-sodium acetate. The column (which retained aspartic and glutamic acids, but not alanine) was washed with water, the washings added to the effluent, and the alanine estimated after confirmation by paper chromatography that no other amino acid was present. Artificial mixtures of alanine, glutamate, and ammonium pyruvate similar to the experimental supernatants were subjected to the ion-exchange separation and gave quantitative recovery of alanine.

The ability of other amino acids to transaminate to pyruvate was investigated qualitatively. Suspensions of the organism were incubated with pyruvate and the appropriate amino acid, and the products examined by paper chromatography. The degree of transamination was judged by the strength of the alanine spot obtained on spraying with ninhydrin. Leucine, tyrosine and arginine were about as active as ammonia itself; valine, phenylalanine, histidine, methionine, less active; tryptophan, serine and threonine, weakly active; proline, lysine, cysteine and glycine gave no appreciable transamination.

Table 6. *Action of inhibitors on alanine synthesis*

Washed suspensions of *Bacillus subtilis* 4 were incubated for 30 min. at 37° with the inhibitor at pH 7. Substrate (ammonium pyruvate) was then added and incubation continued for 3 hr. under standard conditions. The reaction was stopped by addition of HCl. The organisms were removed by centrifugation and the amino-N determined in the supernatants. Final concentrations of inhibitors are recorded in the table.

The following showed no inhibition: sodium cyanide, semicarbazide hydrochloride, hydroxylamine hydrochloride, hydrazine hydrochloride, sodium fluoracetate (all 0.002 M); penicillin, streptomycin, chloromycetin, sulphathiazole (all 0.2 %).

Inhibitor		Q <sub>Alanine</sub>	Inhibition %
None	—	29.0	—
Iodoacetate	0.004 M	5.2	82
	0.0004 M	17.7	39
Sodium fluoride	0.004 M	24.3	16
	0.0004 M	28.4	10
2:4-Dinitrophenol	0.002 M	17.6	39
	0.0002 M	28.5	10
Sodium azide	0.002 M	21.7	25
	0.0002 M	27.8	10
Sodium arsenite	0.002 M	3.5	88
	0.0002 M	13.2	54
8-Hydroxyquinoline	0.001 M	22.3	23
	0.0001 M	28.5	10
None	—	30.5	—
8-Hydroxyquinoline	0.004 M	5.5	82
	0.002 M	10.5	64
None	—	31.8	—
Aureomycin	0.2 %	17.8	44
	0.02 %	26.5	17

*Inhibitors.* The effect of various inhibitors is given in Table 6. The organism was incubated with the inhibitor for 30 min. before adding the substrate (ammonium pyruvate). It is not possible, however, to distinguish between the inhibition of the alanine synthesis itself or the accompanying dissimulation of pyruvate which may be necessary for the reductive amination process.

*Attempts to prepare isolated enzyme extracts.* Study of the mechanism of reductive amination would be much easier if the reaction could be demonstrated in cell-free extracts. But though the system was quite stable in the washed suspensions any interference with the integrity of the cells resulted in severe loss. Thus even lyophilization destroyed 60–80 % of the activity. Attempts at preparing active extracts have so far not been successful.



*Factors determining activity of system*

*Age of culture.* Activity was greatest in growing cultures and declined rapidly when growth ceased (Fig. 4). This raised considerable difficulties when investigating the influence of other cultural conditions, since it was necessary to determine the growth curve in each case in order to harvest at the same stage of growth. The poorer activity of older cultures may be due in part to exhaustion of the medium. Thus, if the organisms are removed from a 24 hr. broth culture and the medium resterilized and re-inoculated, good growth is obtained but the washed suspensions show poor activity. The nature of the substances removed during growth has not been determined. Sewell & King (1955), however, have shown that although the organism will grow on a simple synthetic medium, the reductive amination system does not develop its full activity unless certain amino acids are added to the medium.

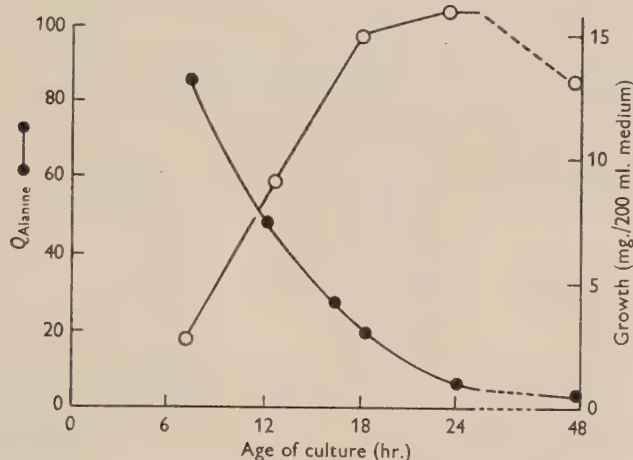


Fig. 4. Relation between age of culture at harvesting and activity of washed suspensions. Organisms grown in Roux bottles on nutrient broth no. 1, harvested after incubation at 37° for time specified, washed suspensions prepared and incubated at 37° for 4 hr. with 0.1 M ammonium pyruvate. Density of each suspension determined by dry-weight determinations.

*pH of growth medium.* Three sources of error had to be avoided: (1) Growth was much slower at the extreme pH values. Hence if all cultures were harvested at, say, 24 hr., it was found that those from acid or alkaline media were most active. This was due to the age-of-culture of effect, the neutral cultures being more mature and therefore less active. If, however, growth curves were prepared for each pH value and the organisms harvested towards the end of the logarithmic phase of growth, optimal activity was obtained over the neutral pH range most favourable for growth (Table 7). (2) A considerable pH shift may occur during growth in nutrient broth. Heavy buffering was undesirable in view of the risk of toxic effects with high salt concentrations, so some change in pH had to be tolerated. The buffer concentrations used and the extent of the pH shift in each case are recorded in Table 7. (3) It is known that

in some cases the pH optimum of an enzyme is dependent on the pH of the growth medium. This was not found to occur in our system.

*Access to oxygen.* The organism cannot grow in the anaerobic jar, so the effect of anaerobiosis could not be studied. Active suspensions could be obtained, however, from cultures grown under forced aeration.

*Temperature.* Here also age-of-culture effects had to be considered. Growth curves were constructed for the organism at both 27 and 37°, and it was found that 39 and 18 hr. respectively represented corresponding physiological ages.  $Q_{\text{Alanine}}$  values of 32 and 30 were obtained for the 18 hr./37° culture and 45 and 40 for the 39 hr./27° culture. The optimum temperature for enzyme formation may be a little below that for growth but the difference is not marked.

Table 7. *Effect of pH of growth medium on activity on suspensions*

Nutrient broth no. 1 was buffered as indicated ('Initial' pH) and inoculated with the organism. After harvesting at the specified time the pH of the medium was determined and recorded ('final' pH). Washed suspensions were prepared and incubated with ammonium pyruvate for 3 hr. at 37° under the standard conditions. The reaction was terminated by addition of HCl, the organisms centrifuged down, and the amino-N determined in the supernatants.

pH of nutrient broth		Buffer	Age at harvesting (hr.)	$Q_{\text{Alanine}}$
Initial	Final			
5.4	5.8	Phthalate, 0.05 M	66	3.2
6.0	6.3	Phosphate, 0.1 M	15	25.5
7.1	7.5	Phosphate, 0.02 M	15	25.2
8.2	8.3	Phosphate, 0.1 M	39	12.5
9.1	8.6	Borate, 0.2 M	63	4.3

*Strain of organism.* Growth curves were determined for the organisms listed in Table 8, using nutrient broth no. 2. Washed suspensions were prepared from cultures harvested towards the end of the logarithmic phase and incubated with ammonium pyruvate under standard conditions. In all cases alanine was the only amino acid detected both before and after acid hydrolysis. This was established by paper chromatography in the two solvent systems. It seems that reductive amination is widely but somewhat irregularly distributed. Two out of six strains of *Bacillus* and the four Gram-negative organisms showed little activity under the standard conditions of test, known to be the most suitable for *Bacillus subtilis* 4. These conditions may not necessarily have been the best for some of the other organisms investigated, and the activities given in Table 8 must be interpreted with this in mind.

*Formation of alanine in growing cultures.* The results described so far were obtained under the somewhat artificial conditions of non-proliferating suspensions. A few confirmatory experiments were made with growing cultures. *Bacillus subtilis* 4 was grown in a mineral medium (no. VII, Stephenson, 1949) with ammonium pyruvate (2%), ammonium lactate (2%), or glucose (2%) as sole organic nutrient. Paper chromatography of the culture filtrates (butanol/acetic acid/butyl acetate system) indicated plentiful alanine production on the pyruvate medium, somewhat less on lactate. On glucose, alanine appeared in

small amounts accompanied by some aspartic acid. The organisms listed in Table 8 (except *Pseudomonas aeruginosa* and *Bacillus megaterium* 7581) were also grown on the pyruvate medium, a very heavy broth inoculum being used to ensure good growth of the nutritionally exacting types. Control experiments showed that this did not introduce detectable amounts of free amino acids into the system. The culture filtrates were examined after several days' growth and all showed plentiful alanine production except *Aerobacter aerogenes*. It will be noted that several organisms which were poor alanine producers in the washed suspensions gave good yield in the growing cultures. In no case was any amino acid other than alanine found in appreciable amount. Certain of these organisms were also grown in the lactate and glucose media. Most gave alanine readily in the lactate medium. With glucose, alanine production was detectable in most cases; moreover (except in *Aerobacter aerogenes*), it was the only amino acid detectable save in very old cultures undergoing autolysis.

Table 8. *Comparative rates of alanine synthesis*

Organisms with NCTC numbers were obtained from the National Collection of Type Cultures: others were stock strains and the numbers refer to our own list. They were grown on nutrient broth no. 2 and harvested at the specified times. These had been determined in each case to be near the end of the phase of active growth. The washed suspensions were incubated with ammonium pyruvate under the standard conditions cited in the text. The reaction was terminated by addition of HCl, the organisms removed by centrifugation and the amino-N-estimated in the supernatants.

Organism	Age at harvesting (hr.)	Gram	$q_{\text{Alanine}}^{\text{N}}$	
<i>Bacillus subtilis</i> 4	16	+	1070	1040
<i>Bacillus subtilis</i> NCTC 3610	16	+	210	140
<i>Bacillus megaterium</i> NCTC 2605	45	+	220	190
<i>Bacillus megaterium</i> NCTC 7581	48	+	40	40
<i>Bacillus mycoides</i> 5	16	+	670	640
<i>Bacillus pumilus</i> NCTC 7576	24	+	30	50
<i>Sarcina lutea</i> 7	45	+	330	240
<i>Sarcina lutea</i> 21	72	+	200	160
<i>Staphylococcus aureus</i> 23	24	+	190	110
<i>Streptococcus lactis</i> NCTC 662	18	+	230	180
<i>Streptococcus faecalis</i> 9	45	+	10	
<i>Mycobacterium phlei</i> NCTC 525	72	(Acid-fast)	190	130
<i>Proteus vulgaris</i> 10	16	—	10	10
<i>Escherichia coli</i> 106	16	—	30	10
<i>Aerobacter aerogenes</i> NCTC 418	18	—	10	10
<i>Pseudomonas aeruginosa</i> 15	16	—	30	10

## DISCUSSION

Two types of mechanism may be considered for this reaction: (1) Indirect, by primary incorporation of the amino group into a dicarboxylic acid either through the mechanism shown to operate in liver slices by Kritzman (1947) or through the reversible glutamic dehydrogenase demonstrated in *Escherichia coli* by Adler, Hellström, Gunther & Von Euler (1938). The amino group would then be transferred to pyruvate by transamination. (2) Direct, not involving any intermediate dicarboxylic acid.



We have not found intermediate formation of aspartic or glutamic acid, as did Kritzman (1947) in liver slices or Nisman *et al.* (1947) in clostridial suspensions. Neither could we demonstrate a requirement for carbon dioxide or dicarboxylic acids. We recognize the difficulty of eliminating these substances when using intact cells. But if *Bacillus subtilis* has a carbon dioxide requirement comparable with that of Kritzman's or Nisman's systems, we could have reasonably expected to detect it. While recognizing that negative results can never be regarded as conclusive, we consider our findings not to be without significance. The observations that alanine was more rapidly formed when ammonia itself rather than aspartate or glutamate was the amino donor, and that neither oxalacetate nor  $\alpha$ -ketoglutarate were as rapidly aminated as pyruvate also introduce the possibility that conversion of pyruvate to alanine might perhaps proceed directly rather than through transamination. We are fully aware, however, that in experiments with intact cells negative results may arise from failure of the reagents to penetrate the cell and that a transamination mechanism might be catalysed by traces of aspartate or glutamate still retained within the washed cells. It is interesting, however, that Shah (1956, unpublished observations) reports that the poor activity of cells grown on a synthetic medium is not enhanced by addition of 0.5% glutamate to the medium. We recognize that our experiments do not exclude the possibility of a transamination mechanism, and that establishment of the mechanism of the reaction may be impossible unless active cell-free extracts can be obtained.

If, however, amination can occur directly this raises the question of the alanine-pyruvate system as a path of entry for the amino group. Rowsell (1951) showed that in animal tissues alanine can participate—without intervention of glutamate—in a wide range of transaminations. It is significant that a wide range of organisms, when grown on glucose, liberate into the medium alanine but no other amino acid. Pyruvate is normally only a transitory intermediate in metabolism but may accumulate under certain conditions and even be liberated into the external medium (Dagley, Dawes & Morrison, 1951).

Since completion of this work, Wiame & Pierard (1955) have presented spectroscopic evidence for a reversible and specific DPN-coupled L-alanine dehydrogenase in a mutant of *Bacillus subtilis*. They show that the oxidative deamination of aspartate, glutamate and glycine proceeds indirectly by transamination with pyruvate followed by oxidative deamination of the alanine thus formed. Since this enzyme is reversible, it provides a potential means of direct alanine synthesis.

We are deeply indebted to Professor R. A. Morton, F.R.S., for his interest and encouragement throughout this work; to the Medical Research Council for assistance in the purchase of materials and equipment and for a personal grant to one of us (A. S. F.); and to the Ministry of Education for an award to C. E. S.

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## Surface Properties Correlated with Sex Compatibility in *Escherichia coli*

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**SUMMARY:** Sex incompatible (F<sup>-</sup>) strains of *Escherichia coli* have a lower acid agglutination point, a stronger affinity for a basic dye and a greater stability in broth cultures as compared with F<sup>+</sup> strains of corresponding phenotype. These findings indicate a correlation between some surface properties and sex compatibility. These surface properties and the F<sup>+</sup> state are transmitted infectively from F<sup>+</sup> to F<sup>-</sup> strains at the same time. Screening for cultural behaviour of infected strains yields results fully consistent with screening for sex compatibility.

In the last few years evidence has been offered for a system of sexual compatibility in *Escherichia coli* K12 (Lederberg, Cavalli & Lederberg, 1952; Cavalli, Lederberg & Lederberg, 1953; Hayes, 1953). On this ground it has been possible to distinguish between self-compatible strains (termed F<sup>+</sup>) defined as those which are productive of recombinants even when crossed with incompatible strains (termed F<sup>-</sup>) which, in turn, have been defined as those which recombine only with F<sup>+</sup> strains. The property F<sup>+</sup> is transmissible from F<sup>+</sup> to F<sup>-</sup> organisms by *infection* due to an agent (F) which has not yet proved separable from the organism.

The nature of F is still unknown mainly because no physiological changes have been so far correlated with changes in F state. However, the great amount of data collected by the authors quoted above does contain useful indications for further investigations in this direction.

Infection seems to be effected by a short contact between single organisms of opposite F state, which modifies properties determining frequency of recombination both relatively and absolutely. In fact, F<sup>+</sup> × F<sup>-</sup> crosses are more productive than F<sup>+</sup> × F<sup>+</sup> ones. The far lower frequency of recombination, compared with that of infection, together with the fact that all recombinants from F<sup>+</sup> × F<sup>-</sup> crosses are F<sup>+</sup>, suggests that infection is a prerequisite of recombination.

On the other hand, F<sup>+</sup> and F<sup>-</sup> seem to be characters resulting from a different degree of the same property, on the ground that in a cross F<sup>+</sup> × F<sup>+</sup> one of the two strains behaves mostly as F<sup>+</sup> and the other as F<sup>-</sup>.

These data, if considered all together for a coherent interpretation, focus the problem of sex compatibility on the factors which determine the contact of organisms. Tentatively, it might be thought that some sort of interaction within the surface of organisms defines the probability and the extent of their contact, besides such other obvious factors as density, agitation, etc., of the suspensions of organisms.

It seems worthwhile, therefore, to search for differences in surface properties between organisms of opposite F state, especially since the finding of properties correlated with sex compatibility might offer new experimental criteria for investigating the problems outlined above. In fact, the screening of F+ and F- strains has been concerned, up to now, only with testing their capacity to recombine with strains of known F state and with ability to transmit F by infection. Both methods, apart from the obvious merit of yielding a direct answer, have the disadvantage of being unsuitable for large-scale experiments.

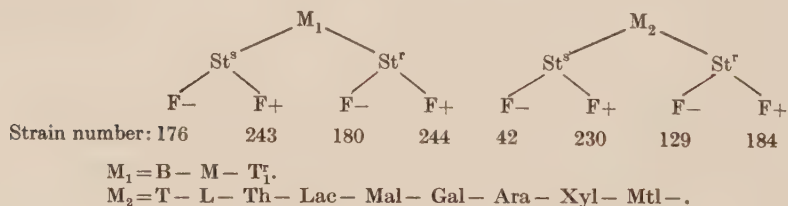
A comparative study of some surface properties of F- and F+ strains has been made by determining: (1) the acid agglutination point; (2) the affinity for acid and basic dyes at different pH values; (3) the stability of broth cultures.

The preliminary results of this investigation (Maccacaro, 1955) were such as to suggest further work of which this paper is the report.

### METHOD

*Organisms.* The strains of *Escherichia coli* used in this investigation were all derivatives of *E. coli* K12 and were kindly supplied by Dr L. L. Cavalli Sforza and Dr L. Fischer Fantuzzi. The phenotype of the strains is fully described in Table 3. Eight of the strains were used more often than the rest, thus permitting factorialization of: (1) two groups of markers, including biochemical deficiencies, sugar fermentations and virus resistance; (2) sensitivity and resistance to streptomycin; (3) F- and F+ state, according to Table 1.

Table 1. *Phenotype and mating type of the 8 strains of Escherichia coli K12 most often used in this investigation*



For the meaning of symbols see Table 3.

*Determination of the acid agglutination point (a.a.p.).* This was carried out by suspending twice-washed organisms in buffers of values ranging from pH 2 to 5 by 0.2 intervals. Buffers were made from 0.1 M-citric acid and 0.2 M-disodium hydrogen phosphate mixed in suitable proportions. Final suspensions contained about  $5 \times 10^7$  organisms/ml.

*Affinity for acid and basic dyes* was tested (Tolstouhov, 1929) by using a mixture of 1 vol. 3.2% (w/v) 'Methylenblau B extra Merck' in pure methanol as basic dye, and 10 vol. of 0.5% (w/v) 'Rubin S extra konzentriert Merck' in pure methanol as acid dye. By addition of 0.1 N-HCl, samples of this dye solution ranging from pH 2 to 5 were prepared and used to stain smears of organisms.

*Stability of broth cultures* was tested nephelometrically in a Zeiss Stufenphotometer, with conical beam of light and filter L1. The medium was either the infusion broth prepared by the Istituto Sieroterapico Milanese (500 g. horse muscle + 20 g. peptone + 5 g. NaCl in 1000 ml. distilled water), or our nutrient broth NB5 (Beef extract Difco 10 g.; Bacto Peptone Difco 10 g.; Nutrient Broth Difco 8 g.; glucose 5 g.; NaCl 8 g. distilled water to 1000 ml.). Both media were adjusted to pH 7.3 and adjusted to the same optical density. Nephelometrical readings were taken, if not otherwise stated, at the 20th hour of incubation.

*Infection* was carried out by mixing the F- and the F+ strains in a large-size test tube containing 5 ml. of one of the two broth media mentioned above. The test tube was then placed in a water bath at 37° and gently shaken throughout the time of infection. When the two strains had been chosen for opposite fermentation marker characters, their subsequent isolation was always made on EMB medium (Lederberg, 1950).

*Recombination* was carried out by inoculating  $10^8$ – $10^9$  washed organisms of each strain on the surface of minimal agar plates (medium of Lederberg, 1947) occasionally supplemented with thiamine.

## RESULTS

### *Determination of the acid agglutination point*

The determination of the acid agglutination point (a.a.p.) was repeated four times for each of the 8 strains studied. Mean values for single strains and for one-factor groups of them are given in Table 2. The statistical analysis of these data shows that the factor most affecting a.a.p. of the studied strains was, by far, the F state ( $P < 0.01$ ). Streptomycin resistance had no effect on a.a.p. ( $P > 0.05$ ), while the effect of the other markers was just significant ( $0.05 > P > 0.01$ ) in the sense that, in terms of a.a.p.,  $M_1$  strains were to  $M_2$  as, broadly speaking, F- strains are to F+. No interaction between these effects is significant.

Table 2. *The acid agglutination point (a.a.p.) of 8 strains of Escherichia coli K 12*

The characters of strains are indicated by the headings of the columns and rows. The datum is the pH value at which agglutination occurs.

	$M_1$	$M_2$	
F- { St <sup>s</sup>	3.000 ± 0.041	2.925 ± 0.050	2.913 ± 0.035
{ St <sup>r</sup>	2.775 ± 0.087	2.950 ± 0.065	
F+ { St <sup>s</sup>	3.350 ± 0.105	3.450 ± 0.076	3.331 ± 0.059
{ St <sup>r</sup>	3.100 ± 0.032	3.425 ± 0.112	
	3.056 ± 0.066	3.188 ± 0.075	

Figures are means and standard errors of four replicates. Marginal means refer to the corresponding one-factor quartet of strains.

The mean a.a.p. for St<sup>s</sup> strains is  $3.181 \pm 0.068$  versus  $3.063 \pm 0.074$  for the St<sup>r</sup> strains.

For the meaning of symbols see Tables 1 and 3.



*Affinity for acid and basic dyes at different pH values*

Staining of the 8 strains with a mixture of Methylenblau B and Rubin S at different pH values resulted in a differential retention of the two stains by the organisms, varying from exclusively Methylenblau B to exclusively Rubin S, according to shifting of the pH values from 4 to 2. The data collected are not yet adequate for a satisfactory analysis, because of some difficulties in standardizing the experimental conditions. Nevertheless, it can be safely said that for any given pair of the studied strains it was possible to find a zone of pH values within the range pH 4-2, at which the F+ variant retained the acid stain, while the F- variant retained the basic one.

*Stability of broth cultures*

The stability of broth cultures of strains with opposite F state was studied nephelometrically. Provided that the light beam impinged on the test tube at about 2 cm. from the bottom, the nephelometer readings were not a function of the total number of organisms in the culture but of the number of them which remained suspended in the medium. In fact, comparable cultures of pairs of strains, identical but for sex compatibility, when shaken gave comparable nephelometer readings and viable counts. As seen from Fig. 1, both F+ and F- strains grew at the same rate as indicated by the nephelometer readings, until a population density was reached at which F+ organisms precipitated while F- organisms did not.

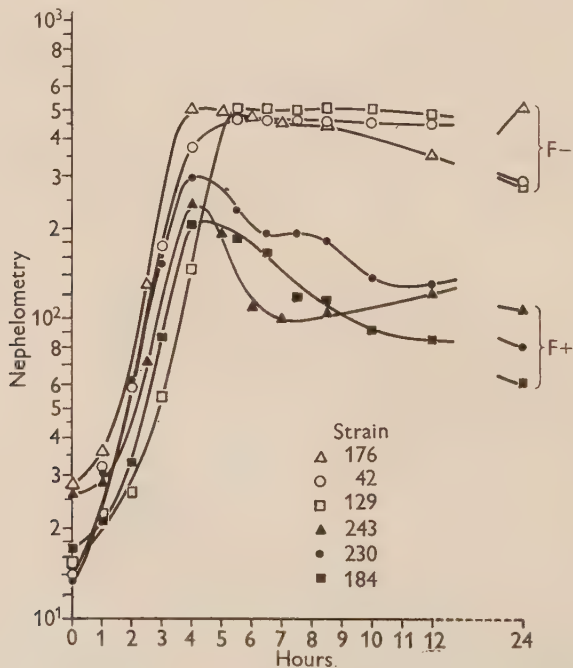


Fig. 1. Broth culture stability of 3 F+ strains (black marks) and 3 F- strains (white marks).  
Medium: infusion broth I.S.M.

Curves given in Fig. 1 are samples chosen from many others and do not represent an extreme situation. On the contrary, a far greater difference between end-points was found very often. It is worth noting that as a rule such curves show some periodicity, just as if the spontaneous precipitation, by clearing the medium, allowed further growth of suspended organisms up to the concentration at which precipitation recurred.

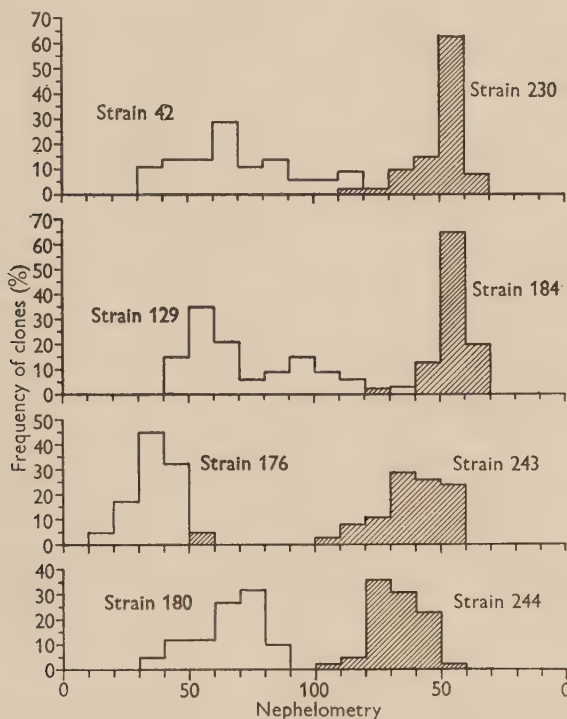


Fig. 2. Interclonal variability of broth culture stability of 8 strains. Shaded histograms relate to F+ variants. Suspension turbidity is recorded in abscissa and decreases continuously from left to right: for sake of simplicity the abscissa values are untransformed readings on the two scales of the Zeiss Stufenphotometer.

Medium: infusion broth I.S.M.

Curves given in Fig. 1 relate to cultures in infusion broth produced by the Istituto Sieroterapico Milanese. They are not reproducible in a poor medium like Nutrient Broth Difco, unless this is suitably supplemented, as is our nutrient broth NB 5.

Table 3 shows the effect of the F state on the stability of broth cultures of 17 pairs of strains with very different phenotypes. The data are recorded as the logarithms of nephelometer readings; it is apparent that for any given pair of strains, the F- strain scores regularly higher, no matter what the phenotype.

However, the extent of such a difference is by no means constant from strain to strain nor, within each pair of strains, is it constant over the two media used. It is our experience that for every strain one should look for the medium which

Table 3. *The effect of F state on the broth culture stability of strains of Escherichia coli K 12 with different phenotypes*

The datum is the log-transformation of a nephelometrical reading.

Strain no.	Mating type	Phenotype	Infusion broth I.S.M.	Nutrient broth NB5
			log nephelometer reading	
256 <sub>B</sub>	F-	Lac- Mal- Gal- Xyl- Ara- Mtl- St <sup>r</sup>	1.734 ± 0.009	2.030 ± 0.029
	F+		1.609 ± 0.019	1.522 ± 0.043
176	F-	B- M- T <sub>1</sub> <sup>r</sup>	2.059 ± 0.022	2.528 ± 0.020
243	F+		1.630 ± 0.006	1.728 ± 0.132
180	F-	B- M- T <sub>1</sub> <sup>r</sup> St <sup>r</sup>	2.581 ± 0.014	2.036 ± 0.023
244	F+		1.570 ± 0.024	1.653 ± 0.010
42	F-	T- L- Th- Lac- Mal- Gal- Ara- Xyl- Mtl-	2.180 ± 0.036	2.423 ± 0.024
230	F+		1.633 ± 0.014	1.464 ± 0.025
129	F-	T- L- Th- Lac- Mal- Gal- Ara- Xyl- Mtl- St <sup>r</sup>	1.787 ± 0.022	2.069 ± 0.026
184	F+		1.460 ± 0.018	1.595 ± 0.024
361	F-	C- Gal <sub>a</sub> - T <sub>1</sub> <sup>r</sup> T <sub>6</sub> <sup>r</sup> St <sup>r</sup> Lp <sup>s</sup>	2.680 ± 0.023	2.522 ± 0.074
	F+		2.308 ± 0.001	2.485 ± 0.041
363 <sub>A</sub>	F-	C- Gal <sub>a</sub> - T <sub>1</sub> <sup>r</sup> T <sub>6</sub> <sup>r</sup> St <sup>r</sup> Lp <sup>+</sup>	2.622 ± 0.044	2.527 ± 0.011
	F+		2.103 ± 0.016	2.457 ± 0.289
365 <sub>C</sub>	F-	C- H- Gal <sub>a</sub> - T <sub>1</sub> <sup>r</sup> Lp <sup>s</sup>	2.645 ± 0.042	2.268 ± 0.165
	F+		1.723 ± 0.019	1.766 ± 0.076
324 <sup>(+)</sup>	F-	H-	2.694 ± 0.019	2.442 ± 0.015
	F+		2.291 ± 0.027	2.352 ± 0.053
367	F-	H- Lac- Ara-	2.808 ± 0.039	2.742 ± 0.030
	F+		2.107 ± 0.030	2.403 ± 0.043
354	F-	H- T <sub>6</sub> <sup>r</sup> Val <sup>r</sup>	2.903 ± 0.036	2.735 ± 0.025
	F+		2.024 ± 0.019	2.104 ± 0.010
368	F-	H- Mal- Gal <sub>a</sub> - T <sub>1</sub> <sup>r</sup> Val <sup>r</sup> Az <sup>s</sup> Lp <sup>s</sup>	2.652 ± 0.039	2.223 ± 0.011
	F+		2.275 ± 0.033	2.151 ± 0.135
369	F-	H- Mal- Gal <sub>a</sub> - T <sub>1</sub> <sup>r</sup> Val <sup>r</sup> Az <sup>r</sup> Lp <sup>+</sup>	2.754 ± 0.016	2.578 ± 0.017
	F+		2.027 ± 0.014	1.676 ± 0.029
337	F-	Pr- T <sub>1</sub> <sup>r</sup> Az <sup>r</sup>	2.870 ± 0.009	2.473 ± 0.018
	F+		2.175 ± 0.033	2.158 ± 0.052
349	F-	Pr- T <sub>1</sub> <sup>r</sup> T <sub>6</sub> <sup>r</sup> Val <sup>r</sup> Az <sup>r</sup>	2.890 ± 0.031	2.688 ± 0.035
	F+		2.295 ± 0.010	2.238 ± 0.018
364	F-	Pr- Gal- T <sub>1</sub> <sup>r</sup> T <sub>6</sub> <sup>r</sup> Val <sup>r</sup> Az <sup>r</sup>	2.745 ± 0.010	2.364 ± 0.073
	F+		2.205 ± 0.027	2.183 ± 0.024
371	F-	Pr- Gal- Ara- T <sub>1</sub> <sup>r</sup> T <sub>6</sub> <sup>r</sup> Val <sup>r</sup> Az <sup>r</sup>	2.951 ± 0.034	2.865 ± 0.032
	F+		2.474 ± 0.016	2.189 ± 0.031

(+) This strain has given inconsistent results in subsequent experiments.

Figures reported are means (± standard errors) of four subcultures.

Meaning of symbols:

Biochemical deficiencies: B=biotin; M=methionine; T=threonine; L=leucine; Th=thiamine; C=cystine; H=histidine; P=proline.

Sugar fermentations: Lac=lactose; Mal=maltose; Gal=galactose; Xyl=xylose; Ara=arabinose; Mtl=mannitol.

Virus resistances: T<sub>1</sub>=bacteriophage T<sub>1</sub>; T<sub>6</sub>=bacteriophage T<sub>6</sub>.

Drug resistances: Az=sodium azide; St=streptomycin; Val=valine.

Lysogenicity: Lp<sup>s</sup>=sensitive to phage λ; Lp<sup>+</sup>=lysogenic for phage λ.



yields the maximum difference in broth culture stability between the F- and the F+ variants of the same phenotype. Accordingly, a statistical analysis of data reported in Table 3, besides showing the impressive effect of F ( $P < 0.001$ ) reveals a highly significant effect of the medium. Moreover (Table 4), the latter is significantly interacting with the effect of F which is, on the whole, appreciably greater in the infusion broth I.S.M. than in nutrient broth NB5. Also the interaction 'F  $\times$  phenotype' is significant.

Table 4. *General means ( $\pm$  standard error) of data collected in Table 3*

	F-	F+	
Nutrient broth NB5	2.442 $\pm$ 0.032	2.007 $\pm$ 0.045	2.225 $\pm$ 0.034
Infusion broth I.S.M.	2.562 $\pm$ 0.046	1.995 $\pm$ 0.038	2.279 $\pm$ 0.038
	2.502 $\pm$ 0.028	2.001 $\pm$ 0.029	

The two main effects: 'F' and 'medium' and their interaction are highly significant ( $P < 0.001$ ).

The 8 strains on which the acid-agglutination point was determined were submitted to a more extensive study of their cultural pattern: 40 clones of each strain were examined nephelometrically (Fig. 2).

The mean values of the log-transformation of nephelometrical readings are: 2.070  $\pm$  0.008 for the M<sub>1</sub> strains versus 1.918  $\pm$  0.009 for the M<sub>2</sub> strains; 2.030  $\pm$  0.009 for the St<sup>a</sup> strains versus 1.958  $\pm$  0.007 for the St<sup>r</sup> ones; 2.247  $\pm$  0.010 for the F- strains versus 1.740  $\pm$  0.007 for the F+ ones. All these differences are highly significant ( $P < 0.01$ ). The interaction 'markers  $\times$  streptomycin resistance' is irrelevant; the interaction 'streptomycin resistance  $\times$  F' is highly significant ( $P < 0.01$ ) in the sense that the effect of F is greater in St<sup>a</sup> strains.

#### *F+ and F- in rough and smooth forms*

It has long been known that broth cultures of bacteria in the rough form are very much more unstable than smooth cultures. Therefore, the question arises as to how much of the difference in culture stability between F+ and F- strains is due to a morphological difference. In fact, F- strains, when plated on nutrient agar, produce a majority of smooth colonies (about 70-90%) which is decreased to a minority (about 20-40%) in F+ strains.

Moreover, there is the same shift in the distribution of smooth colonies during the course of infection of an F- strain by an F+ one. But data of this type are inadequate to give a quantitative knowledge of the impact of rough/smooth dissociation on the cultural pattern of compatible and incompatible strains. Therefore a number of subcultures from rough and smooth colonies (care was taken to discard colonies of intermediate morphology) were studied nephelometrically. The investigation was made on all 8 strains quoted above with an experimental design enabling factorialization of: (1) F state; (2) rough/smooth form; (3) streptomycin resistance. Part of the results of these experiments are shown in Fig. 3. Averages calculated on the full set of experimental results are reported in Table 5. A statistical analysis of these data shows that,

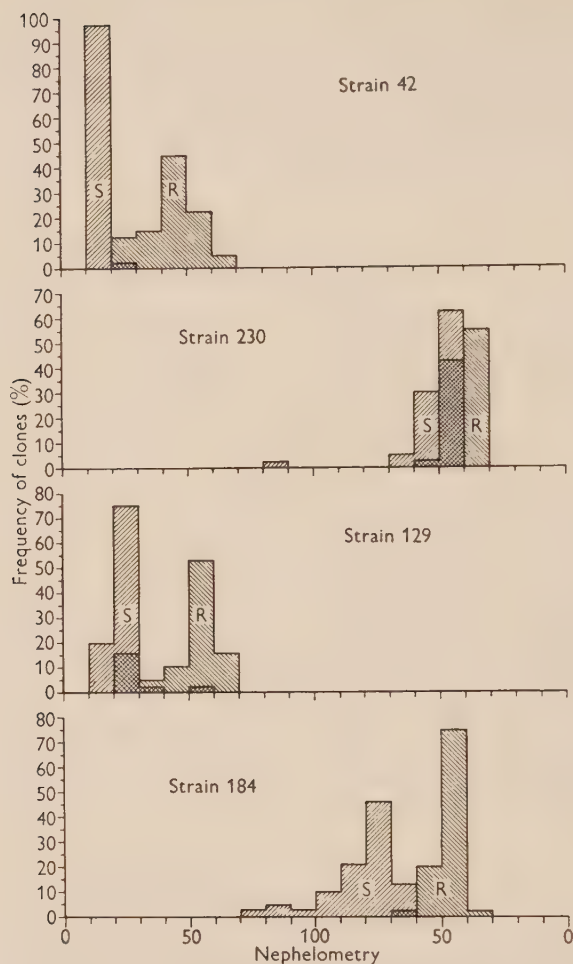


Fig. 3. The impact of S-R dissociation on the broth culture stability of F+ and F- strains. Medium: nutrient broth NB5.

Table 5. The impact of *F* state (F-/F+), morphological form (rough (R) or smooth (S)) streptomycin resistance (*St*<sup>s</sup>/*St*<sup>r</sup>) on the stability of broth cultures of *Escherichia coli* K12.

The datum is the log-transformation of a nephelometric reading. Figures reported are means and standard errors of 80 subcultures of which 40 are from a TLB<sub>1</sub>- strain and 40 from a BM-. The medium was nutrient broth NB5.

	F-	F+	
S {	<i>St</i> <sup>s</sup>	2.761 ± 0.011	2.121 ± 0.028
	<i>St</i> <sup>r</sup>	1.671 ± 0.009	
R {	<i>St</i> <sup>s</sup>	2.310 ± 0.044	1.883 ± 0.020
	<i>St</i> <sup>r</sup>	1.740 ± 0.024	
R {	<i>St</i> <sup>s</sup>	2.287 ± 0.021	1.609 ± 0.008
	<i>St</i> <sup>r</sup>	1.609 ± 0.008	
		1.583 ± 0.013	
	2.353 ± 0.021	1.651 ± 0.008	

The mean value for *St*<sup>s</sup> is 2.082 ± 0.027; that for *St*<sup>r</sup> is 1.922 ± 0.022.

besides the main effects just mentioned, namely, (1) mating type; (2) rough/smooth form; (3) streptomycin resistance (F, Ph and St), the following interactions of first and second order are also highly significant ( $P < 0.001$ ): (1) the effect of F is greater in smooth, i.e. the effect of Ph is greater in F +; this interaction is greater in St<sup>+</sup>; (2) the effect of F is greater in St<sup>+</sup>, i.e. the effect of St is greater in F -, this interaction is greater in smooth; (3) the effect of Ph is greater in St<sup>+</sup>, i.e. the effect of St is greater in smooth; this interaction is greater in F -. On the whole it is clear that broth culture stability is affected by F state and rough/smooth state quite independently.

*The cultural behaviour as a co-ordinate for the time-course of infection*

Provided the difference in F state is highly correlated with the stability of broth cultures and that morphological dissociation cannot account for such correlation, it seemed legitimate to attempt a description of the kinetics of F infection in terms of cultural behaviour. With this aim, strain 176 (F - Lac +) has been infected with strain 230 (F + Lac -) under experimental conditions already described. The mixture of the two strains was plated at intervals on EMB, whence 40 colonies were transferred to infusion broth I.S.M. for a test of cultural stability. Results are shown in Fig. 4.

Analogous results have been obtained with infection of strain 176 by strain 184, of strain 42 by strains 243 and 244, of strain 129 by strains 243 and 244, of strain 180 by strains 230 and 184.

When colonies of the donor strain were isolated at intervals during the infection and used for a test of cultural stability no modifications of this property were detected in them.

Particular attention was given to the effect of changes in experimental conditions on the kinetics of the transformation of cultural behaviour, with the aim of establishing what degree of standardization would be needed in further researches. It was found that this transformation is not appreciably affected by variations of temperature between 35 and 39° nor by variations of pH between 6.4 and 7.5. On the contrary it is strongly enhanced by agitation of the mixture of organisms. Transformation of the cultural behaviour was not obtained when infection was attempted in distilled water, saline and minimal medium unless these media were supplemented with yeast extract (0.5%). The effect of the initial ratio of the number of F - organisms to that of F + was tested by simultaneous infections of strain 176 with strain 230 at different F - to F + ratios ranging from 1:8 to 8:1 on a twofold scale. Results are given in Fig. 5 and clearly show the importance of the relative concentration of the donor and the acceptor strains. In fact, when 40 clones of strain 42 and strain 176, infected respectively with strains 243 and 230, were screened as F + and F - on the basis of their cultural behaviour and then submitted to recombination tests, results were fully consistent.



## DISCUSSION

The main result of this investigation is the detection of properties other than compatibility which are correlated with the F+ state, at least as far as our strains of *Escherichia coli* are concerned. These properties, namely: cultural

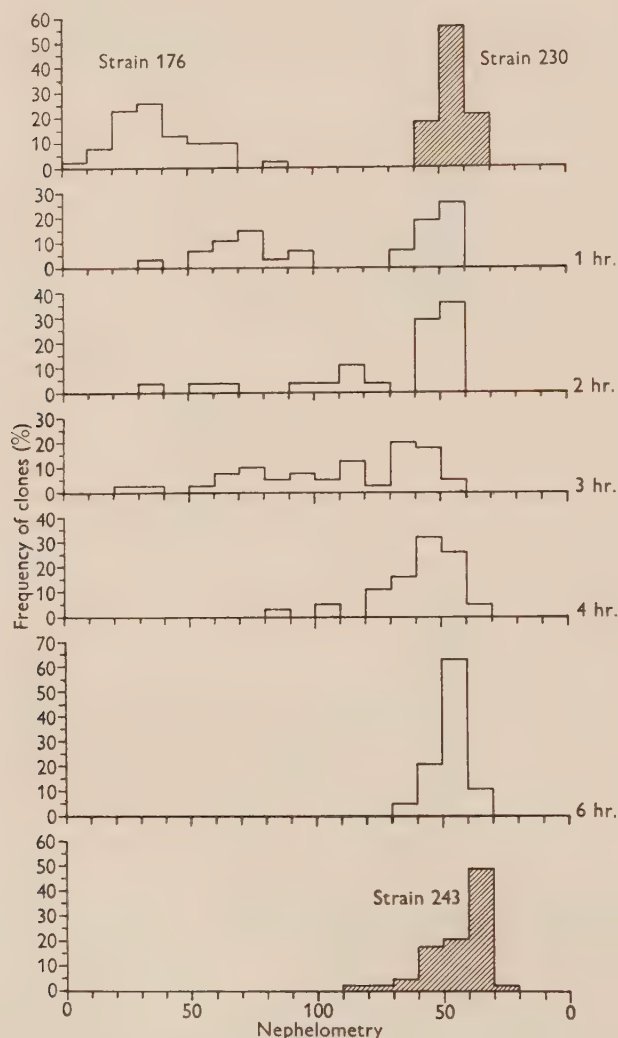


Fig. 4. The time-course of the infective transformation of broth culture stability. Strain 176 (F-) has been infected by strain 230 (F+) to obtain strain 243 (F+). Medium: infusion broth I.S.M.

behaviour, acid agglutination and affinity for stains, are open to a unitary interpretation in terms of the surface properties of the bacteria. In fact, all experimental results indicate very coherently that the F- organisms behave as if they were more negatively charged than F+.

Of course, whether homogeneous dispersions or agglutination occur, either spontaneously or because of a critical potential, must depend upon the balance of the cohesive and repulsive forces which are related to many factors, like hydration of ionizing and non-ionizing polar groups, adsorption of ions at the surface of the organism, proper electric charges of the organisms, etc. (Lamanna & Mallette, 1953). Therefore, at this stage of our knowledge, to speak in terms of surface charges has no other validity than that of a simplification making easier the shaping of a model, which could be stated as follows.

Between bacteria suspended in a medium, cohesive and repulsive forces are produced, according to their surface charges. Sex compatibility of two strains is influenced by this interaction which is defined by the relative no less than by the absolute values of the charges. Self-compatible strains differ from the others in a surface structure which can be induced by contact (infection).

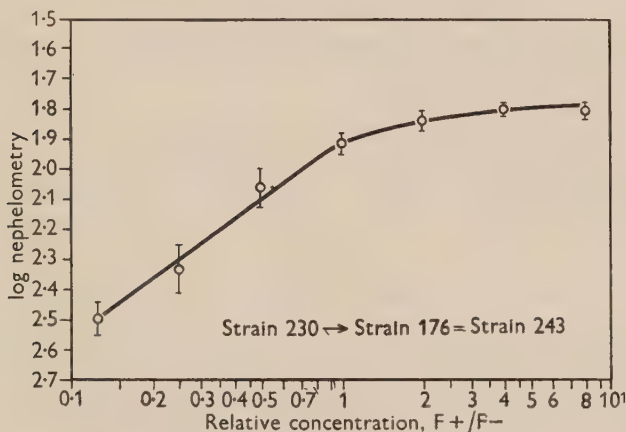


Fig. 5. Effect of varying the ratio of  $F^+$  to  $F^-$  cells on the efficiency of transformation of broth culture stability. Small circles each represent means ( $\pm$  standard error) of forty data. Medium: nutrient broth NB5.

All experimental results here reported are in agreement with this model but, unless supporting proofs of a more direct nature can be offered, the mere observation of a correlation of sex compatibility and surface properties is no reason to accept a cause-and-effect relationship. On the other hand, some supporting data are not to be dismissed:

(1) Various  $F^+$  strains have different  $F^+$  strengths and the relative behaviour of the two strains depends on the difference of  $F^+$  strengths between the two (Cavalli *et al.* 1953).

(2)  $BM^-$  strains which have a mean acid agglutination point significantly lower than that of  $TLB_1^-$  strains are weaker  $F^+$  than the latter (Table 2 of this paper; and Cavalli *et al.* 1953).

(3) Electrophoretic mobility drops to a minimum (Abramson, Moyer & Gorin, 1942) and frequency of recombination rises to a maximum (Maccacaro, 1953) during the early phases of growth of a culture.

(4) Oxygenation of a BM- F+ culture converts it into a reversible F- phenocopy (Lederberg *et al.* 1952). Exposure of cultures of *Staphylococcus pyogenes* to oxygen prevents the effect of agents which would make the organisms less electronegative (Lerche, 1953).

(5) No morphological equivalents of sex compatibility have been detected at the cell surface by electron microscopy (Maccacaro & Pasinetti, 1955).

Needless to say, many other facts cannot be fitted at this stage, within the model sketched above. But in whatever way the available evidence is taken, to say that compatibility and surface properties are highly correlated seems a safe conclusion, resting on the following points which seem proved beyond any doubt:

(1) Between the F- and the F+ variants of every tested strain there is a uniform difference in surface properties.

(2) Compatibility and surface properties are transmitted infectively from F+ to F- strains along the same time-course.

(3) Screening of infected strains for cultural behaviour yields results fully consistent with scoring for compatibility.

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## Heterokaryosis and Parasexual Recombination in Pathogenic Strains of *Fusarium oxysporum*

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**SUMMARY:** Mutants of *Fusarium oxysporum* f. *pisi* (cause of pea wilt), distinguished from the parent types by their different morphology, pathogenicity and nutritional requirements, were obtained by ultraviolet irradiation. Pairs of mutants with different nutritional requirements (auxotrophs) formed balanced heterokaryons on non-supplemented medium. Most of the auxotrophs were less pathogenic than the wild-types, whereas heterokaryons between the mutants were not.

Single conidia of a heterokaryon between an auxotroph from race 1 and one from race 2 of *Fusarium oxysporum* gave a small proportion ( $3 \text{ in } 10^8$ ) of colonies which were able to grow on non-supplemented medium. These three prototrophs were presumably diploid because vegetatively they gave new strains with various combinations of colour, nutritional requirements, actinomyecete tolerance and pathogenicity.

The results indicate that, in fusaria, heterokaryosis plays a part in variation of virulence and that *Fusarium oxysporum*, which has no known sexual stage, has a system similar to the parasexual cycle described by Pontecorvo for certain other Fungi Imperfecti, that permits the segregation and recombination of genetic factors outside the sexual stage.

Many plant pathogenic fungi which seem to have no perfect stage nevertheless vary greatly in their pathogenicity. Analyses of laboratory mutants of some Fungi Imperfecti by Pontecorvo, Roper & Forbes (1953) and by Pontecorvo & Sermonti (1954) showed how genetical techniques can be applied to imperfect fungi. Heterokaryons (colonies with hyphae containing nuclei of different genetic types) regularly produced rare diploid nuclei, and characters recombined in new ways in the subsequent mitotic divisions. That heterokaryosis could affect the virulence of fungi was shown by Hansen (1938), who worked with *Phoma terrestris* and *Botrytis cinerea*.

The present paper describes experiments made to test whether or not heterokaryosis and a system of recombination occur in *Fusarium* spp. and could explain some of their variability in pathogenicity. *F. oxysporum* Fr. includes soil-borne pathogens which cause serious diseases of crops such as banana, flax, peas and tomatoes. Buxton (1954) showed that culturally distinct isolates of *F. oxysporum* Fr. f. *gladioli* Snyder & Hansen (cause of gladiolus yellows) form heterokaryons on agar media. As usual, when these fungi are exposed to ultraviolet radiation, mutants occur which differ from the parent type and from one another in their requirements for growth factors. Pairs of nutritional mutants (auxotrophs) may be able to form heterokaryons with each other, heterokaryons which can grow on media devoid of the growth factors necessary for mutants themselves.

Auxotrophic mutants, produced by ultraviolet radiation, were used in the

work described below because morphological features in fusaria are unreliable as genetic markers and are rarely related to differences in pathogenicity, either the plants attacked or the severity of the disease caused. *F. oxysporum* f. *pisi* (cause of pea wilt) is suitable for this type of work because it grows quickly in culture and produces many conidia on agar media. Moreover, peas are easy to grow in quantity and disease symptoms appear quickly.

## RESULTS

*Production of nutritional and morphological mutants.* Three isolates were used, grown from single conidial cultures derived from colonies isolated from diseased peas: *Fusarium oxysporum* f. *pisi* race 1 (American isolate from wilted peas); *F. oxysporum* f. *pisi* race 1, 612A (English isolate from wilted peas); *F. oxysporum* f. *pisi* race 2 (American isolate from 'near-wilted' peas).

Conidia were irradiated with ultraviolet light from a Hanovia XII medium pressure lamp, 95 % of the irradiation being of wavelength 2537 Å., giving  $173 \mu\text{W./cm.}^2$  at 40 cm. distance. Specific nutritional requirements, detected by the usual techniques (Pontecorvo, 1953), are shown in Table 1. Before auxotrophs produced in this way were used in experiments, cultures were established from single conidia.

Table 1. *Auxotrophs and morphological mutants obtained by ultraviolet irradiation of Fusarium oxysporum* f. *pisi*

Strain irradiated	Conidia irradiated (1000/dish) to give 10 % survival	Colonies isolated (from 10 % survival level)	Mutants obtained after screening tests				
			Nutritional		Morphological		
			Amino acid requirers	Nucleic acid requirers	Dwarfs		Semi- dwarfs and slow- growers
					White	Red	
<i>F. oxysporum</i> f. <i>pisi</i> race 1	45,000	1109	18	5	4	0	10
<i>F. oxysporum</i> f. <i>pisi</i> race 1 (612A)	32,000	726	12	4	4	2	17
<i>F. oxysporum</i> f. <i>pisi</i> race 2	8,000	284	1	1	0	0	1
Total	85,000	2119	31	10	8	2	28

*Formation of heterokaryons in culture.* Six of the auxotrophs were selected, and attempts were made to form balanced heterokaryons between pairs of them by streaking the mixed spores on a minimal medium (Pontecorvo, 1949) on which either alone has a lag period and then shows limited growth. Heterokaryons showed within a few days as fans of faster-growing mycelium. All pairs of the mutants of *Fusarium oxysporum* f. *pisi* race 1 (American isolate) and *F. oxysporum* f. *pisi* race 1, 612A, formed balanced heterokaryons and had growth rates approximately equal to that of the wild-types.

Table 2 shows the types of the mutants used in the experiments. In addition, 21 dwarf mutants, with colour differences but no detectable nutritional deficiencies, readily formed heterokaryons whose growth rates were greater than those of the dwarfs, but only half those of the wild-types.

Table 2. *Origin and type of Fusarium mutants used in the inoculation experiments*

Code no.	Origin	<i>In vitro</i> characteristics	
		Nutritional requirement	Morphological appearance
C25	<i>F. oxysporum</i> f. <i>pisi</i> race 1, 612A (English isolate)	Methionine	Bushy
K27	<i>F. oxysporum</i> f. <i>pisi</i> race 1, 612A (English isolate)	Yeast nucleic acid	Dendritic
H20	<i>F. oxysporum</i> f. <i>pisi</i> race 1 (American isolate)	Asparagine	Normal
H32	<i>F. oxysporum</i> f. <i>pisi</i> race 1 (American isolate)	Arginine	Normal

*Inoculation experiments.* The virulence of auxotrophs was compared with that of balanced heterokaryons formed between them. Pea seedlings of the variety Onward (susceptible to wilt) were raised in boxes of unsterilized John Innes compost, and later inoculated as described by Buxton (1955). The pots of inoculated plants were stood in peat maintained at 21° and illuminated (fluorescent 40 W. tubes) with 340 f.c. at soil level for 3 hr. each day at dusk. Disease incidence and disease rating (the ratio of wilted leaves to total leaves per plant, expressed as a percentage) were recorded at weekly intervals.

Table 3. *Disease ratings of peas inoculated with nutritional mutants, heterokaryons and wild-types of Fusarium oxysporum f. pisi*

	Single strains	Heterokaryons
<i>F. oxysporum</i> f. <i>pisi</i> 612A	100.0	—
<i>F. oxysporum</i> f. <i>pisi</i>	100.0	—
No inoculum	0	—
H32 (arginine requirer)	62.5	96.3 87.5 100
K27 (yeast nucleic acid requirer)	40.0	
C25 (methionine requirer)	47.5	
H20 (asparagine requirer)	83.7	

The figures are the means of the results of six identical experiments, seventy-two replicates in all, and are significant at 0.1 % level.

The two auxotrophs of *Fusarium oxysporum* f. *pisi* race 1, 612A, tested were less than half as virulent as the wild-type or heterokaryon. The two autotrophs of *F. oxysporum* f. *pisi* race 1 (American isolate) tested were also less virulent than their parent type. When the heterokaryons were used as inoculum, their virulences were equal to those of the wild-types, a stimulus probably caused by complementary gene action analogous to that restoring growth rate on minimal medium *in vitro*.

Nutritional deficiencies may decrease the pathogenicity of mutants by preventing either penetration of the host-plant root or growth within it. Plants inoculated with mutants C25 (methionineless, bushy) or K27 (yeast nucleic acid-less, dendritic) were therefore treated with a solution of the required nutrient 4 days after transplanting. The nutrients were either added to the soil near the roots or directly injected into the stem base 2 cm. above soil



level. After adding the nutrients to the soil, the pathogenicity of both mutants was restored to about 80 % of that of the wild-type, whereas injection into the stem restored the pathogenicity to only 60 % of that of the wild-type.

To find whether the mutant, wild-type or heterokaryon had been modified during passage through the plants, pieces of the stele and cortex of stem bases and roots from inoculated plants were plated on agar media. From plants inoculated with either mutants or heterokaryons the fungus used as inoculum was recovered and identified. Most of the heterokaryons retained their original form, as shown by culturing single conidia from them, but a few hyphae that grew from plants inoculated with heterokaryons contained only one or other of the mutant-type nuclei from which the heterokaryon was originally synthesized.

*Segregation and recombination between physiologic races.* Pontecorvo (1954) emphasized that heterokaryosis alone cannot give rise to strains with new hereditary constitutions. The experiments described above suggest that, as expected on genetical theory, increased virulence can arise as a phenotypic result of heterokaryosis between two mildly virulent strains. It seemed important to find whether or not, in addition, any system of recombination between physiologic races occurred in *Fusarium oxysporum*.

To investigate this possibility, conidia of single mutants of physiologic races 1 and 2 were re-exposed to ultraviolet radiation to obtain multiple mutants of each race. Twelve multiple mutants, requiring specific amino-acids, were tested for pathogenicity and one from each race finally selected because it retained its wild-type pathogenicity. These two were further characterized by their actinomycete tolerance (Buxton & Richards, 1955) and by their colour on modified Czapek-Dox agar (less  $\text{NaNO}_3$ ) when incubated in darkness at 25°. Balanced heterokaryons were formed between them and single conidial suspensions were spread on a minimal agar medium to detect possible diploids (Roper, 1952; Pontecorvo *et al.* 1953). From  $10^8$  conidia, three proved to be prototrophs on the minimal medium and were subcultured as single conidia to minimal agar slants. When 360 single conidia of these prototrophs were cultured on augmented minimal medium, twelve showed segregation or recombination of the characters of the original heterokaryon (Table 4). The six that differed most from the two original components of the heterokaryon were then tested for their pathogenicity towards three pea varieties that differentiate the three physiologic races of *Fusarium oxysporum* f. *pisi* (Schreuder, 1951; Buxton, 1955), to see whether factors determining race specificity could combine in new ways with each other and with other known 'markers'.

Nearly all recombinants retained the white colour of the original component E6/12 on the modified Dox agar. Strains 5 and 6 were prototrophic but no longer red. Actinomycete tolerance, which was lost by the heterokaryon B28/1 + E6/12, was regained by strains 8 and 11. The pathogenic abilities of the mutants, which were tested in replicated experiments on three different occasions, showed an equally high degree of recombination with the auxotrophic properties. In strain 8, pathogenicity was lost completely, whereas

strains 5 and 10 had race 2 type pathogenicity, identical with that of E6/12. Three of the strains (1, 3 and 9) affected var. Delwiche Commando, although only one of them, strain 3, also wilted var. Alaska.

That the prototrophs were probably diploid may be inferred from the fact that they grew like wild-types on minimal agar, and that they subsequently produced segregants and recombinants. Table 4 shows that this occurred both in cultural and in pathogenic properties. Furthermore, spore measurements

Table 4. *Nutritional mutants used in the synthesis of heterokaryons, and segregation and recombination in Fusarium oxysporum f. pisi races 1 and 2*

Colonies tested	Nutritional 'markers' (as growth responses on augmented minimal media)							Colour on modified Czapek-Dox agar	Actino-mycete tolerance	Pathogenicity on pea varieties		
	Arg.	Asp.	Cyst.	Glut.	Meth.	Pro.	Vit. B <sub>1</sub>			On-ward	Alaska	Del-wiche Com-mando
Race 1	—	—	—	—	—	—	—	White	+	+	—	—
Race 2	—	—	—	—	—	—	—	Red	—	+	+	—
B28/1 (ex race 1)	—	—	+	—	+	—	+	Red	—	+	—	—
E6/12 (ex race 2)	+	+	—	+	—	+	—	White	+	+	+	—
Heterokaryon B28/1+E6/12	—	—	—	—	—	—	—	Red	—	+	—	—
Prototroph B28/1/E6/12	—	—	—	—	—	—	—	Red	—	+	+	—
Recombinants and segregants from prototroph:												
1	—	—	—	—	—	—	—	White	—	+	—	+
2	+	—	—	—	+	+	—	Red	—	—	Not tested	—
3	+	I	—	—	+	+	—	Red	—	+	+	+
4	—	—	—	—	+	+	—	Red	—	—	Not tested	—
5	—	—	—	—	—	—	—	White	—	+	+	—
6	—	—	—	—	—	—	—	White	—	—	Not tested	—
7	+	—	—	—	+	+	—	White	—	—	Not tested	—
8	+	—	—	—	+	+	I	White	+	—	—	—
9	+	—	—	+	+	+	—	White	—	+	—	+
10	+	I	—	—	+	+	—	White	—	+	+	—
11	+	—	—	—	+	+	—	White	+	—	Not tested	—
12	+	—	—	—	+	+	—	White	—	—	Not tested	—

Arg.=arginine; Asp.=asparagine; Cyst.=cystine; Glut.=glutamine; Meth.=methionine; Pro.=proline; Vit. B<sub>1</sub>=vitamin B<sub>1</sub>; I=inhibition. Cyst., meth. and vit. B<sub>1</sub> are all required by B28/1; arg. and asp. are both required by E6/12, but glut. and pro. are alternatives to asparagine.

showed that the diameters of the prototroph microconidia, which are uni-nucleate (Buxton, 1954), were 1.23 times those of both original mutants, B28/1 and E6/12. All this suggests that *Fusarium oxysporum f. pisi* has a system comparable to the parasexual system described in *Aspergillus niger* (Pontecorvo *et al.* 1953) and in *Penicillium chrysogenum* (Pontecorvo & Sermonti, 1954). This system may explain many, but by no means all, of the morphological changes fusaria undergo in culture and of changes in pathogenicity and saprophytic ability of wild-types. A genetic analysis of pathogenicity in *F. oxysporum*, where no perfect stage is known, might be possible by the methods recently outlined by Pontecorvo & Kafer (1954).

## DISCUSSION

Fusaria vary greatly in morphology and in pathogenicity, and many workers have attempted to establish a relationship between a particular cultural type and a defined pathogenicity. Borlaug (1945) showed that several 'biotypes', differing in pathogenicity, occurred in his isolates of *Fusarium lini*. Cormack

(1951) recorded variability in virulence of isolates of *F. avenaceum* and *F. arthrosporioides* and showed that it was, to a limited extent, related to differences in cultural morphology. Oswald (1949) found that two types of fusaria from cereals with root-rots differed morphologically and in virulence. None of these authors considered how fusaria maintain variability in nature, although Snyder (1933) suggested that heterokaryosis might be important in cultural variation, and Leonian (1930) considered that dissociation in fusaria might be due to an undiscovered process which he called 'amorphous sexualism'.

From the many forms of fusaria in the soil, those are selected which can attack the host plant. The experiments described in this paper suggest how these separate pathogenic units could be combined in heterokaryons to produce units with increased virulence.

The ability of weak pathogens to enhance virulence by forming heterokaryons obviously offers a better chance of survival, for weak parasites would thus be given better opportunity to invade a living host and avoid competition with saprophytic soil organisms. Similarly, any deficiencies that limit saprophytic growth in soil might be overcome by heterokaryons forming between strains with different metabolic capabilities. The advantage endowed by heterokaryosis, however, is limited by the pool of genetic potentialities already existing within particular fusarium populations in the soil. With heterokaryosis alone, no recombination of these characters is possible, and, indeed, the system can only increase the competitive ability of fusaria by bringing together in a common cytoplasm nuclei from two strains which are poorly virulent but for different physiological reasons (Pontecorvo, 1954), and this might result in increase or decrease of parasitic ability. The existence of a parasexual cycle enhances the possibilities of survival of the different physiologic races when only unsuitable host varieties are available for infection. In diploids, heterozygosity could be perpetuated and their potentiality for segregation and recombination would enable new hosts to be invaded or new soil environments to be tolerated. That diploids can give rise to new types of pathogenicity, as shown by the ability of some of these new types to infect var. Delwiche Commando, a differential host for race 3, does not imply, of course, that race 3 arises in nature from races 1 and 2.

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## Transduction of Virulence within the Species *Salmonella typhimurium*

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**SUMMARY:** Four strains of *Salmonella typhimurium* avirulent for mice were found to be capable of acting as gene acceptors in transduction experiments. An attempt was made to transduce virulence to three strains, using a typical mouse-virulent strain of *S. typhimurium* as donor. Two of the strains which are adenine-dependent were successfully made virulent by transduction and simultaneously became adenine-independent. The other strain M206, whilst possessing the typical antigenic structure of *S. typhimurium* and being non-exacting nutritionally, was not made virulent by transduction.

Although most strains of a bacterial species behave similarly in their virulence for various animals, there are numerous examples of bacterial strains which are atypical in this respect. Bacterial virulence may be dependent on a number of factors and the absence of any one of these may result in loss of virulence. With bacterial strains which are capable of genetic recombination it may be possible to transfer the missing factor from a virulent to an avirulent strain, thus making the latter virulent. This was done in the case of the pneumococcus by Griffith (1928); rough avirulent non-capsulated strains were transformed into smooth virulent capsulated ones, confirming the importance of the capsule for virulence in this species. The present paper deals with attempts to transduce virulence within the species *Salmonella typhimurium*.

### METHODS

**Cultures.** Two virulent strains of *Salmonella typhimurium* were used throughout. Both were smooth strains, and Felix has shown by quantitative agglutination tests (see Felix & Pitt, 1951) that they are similar both in their 'O' and heat-labile antigens. One strain, C5, was phage typed by Dr E. S. Anderson and belongs to phage type 2A (Felix & Callow, 1943; Felix, 1956). The other, LT2, is the propagating strain of *S. typhimurium* for phage PLT22 (Zinder & Lederberg, 1952).

Four avirulent cultures were obtained. The avirulent *Salmonella typhimurium* M206 was reported by Dr E. S. Anderson as phage type I (Felix & Callow, 1943; Felix, 1956). It was smooth and similar to C5 and LT2 quantitatively as regards its 'O' and heat-labile antigens. Moreover, it was able to grow on minimal medium (Davis & Mingioli, 1950). Its avirulence cannot therefore be attributed to its antigenic structure or to a biosynthetic deficiency. It was a mutant from a fully virulent culture from which it differed in virulence and by being sensitive to complement *in vitro* (Maaløe, 1948).

The other three cultures, D2, D10 and 1901 were adenine-dependent strains of *S. typhimurium*. They were obtained by independent and non-allelic mutations. Both virulent and avirulent strains were able to accept genetic material conveyed by phage PLT22. All cultures were incubated at 37°.

**Bacteriophage.** Suspensions of phage PLT22 (Zinder & Lederberg, 1952) were used for all transduction experiments. The phage was propagated twice on each new host to ensure that few phage particles carrying the genotype of the original host were present. The optimum concentration of phage particles for transduction was  $5 \times 10^9$ /ml. Phage suspensions containing over  $10^{10}$  particles/ml. were obtained by propagation in sloppy agar. The clarified lysates were heated at 57° for 1 hr. and tested for sterility. The phage titre was obtained by plaque count by the soft agar layer method (Gratia, 1936), using *Salmonella gallinarum* SL140 as indicator strain.

**Test animals.** Mice (18–22 g.) of a pure line of Swiss white mice no. 1 were used.

**Virulence tests.** The evaluation of any results depended solely on differences in the virulence of the strains. It was therefore essential to standardize virulence tests in order to obtain reproducible results. Cultures were grown overnight in tryptic digest broth, diluted 1/1000 in warmed tryptic digest broth and 2 ml. of this dilution incubated for a further 6 hr. Tenfold dilutions of this culture were prepared in saline and 0.2 ml. of each dilution injected intraperitoneally into each of five mice. The number of viable organisms injected was ascertained from counts prepared in triplicate from the same dilutions, by the drop method of Miles & Misra (1938). In this medium the number of viable organisms of the avirulent *Salmonella typhimurium* M206 averaged  $1.5 \times 10^9$ /ml. after overnight and  $6.5 \times 10^8$ /ml. after 6 hr. incubation of the diluted culture. Deaths from *S. typhimurium* usually occurred between the 5th and 21st day and injected mice were observed up to 30 days. After death heart blood was plated out on nutrient agar and on MacConkey's bile salt lactose agar, and incubated overnight. The identity of the culture was confirmed by slide agglutination.

**Calculation of LD50.** The approximate LD50 was calculated by the method of Reed & Muench (1938). A single experiment with five mice at each dose level would result in only approximate results, but the virulence of each strain has been confirmed frequently at the critical levels and no large differences have been noted.

**Transduction of virulence.** Two methods of transduction differing only in detail were used.

To 1 ml. of an overnight broth culture of the avirulent strain diluted either 1/10 or 1/100 in warmed broth was added 1 ml. of phage suspension (titre  $5 \times 10^9$  particles/ml.) which had been harvested from the virulent parents LT2 or C5; the mixture was incubated for 4 hr.

Alternatively, 3 ml. of overnight culture were diluted with 9 ml. broth and incubated for 1 hr. To 1 ml. of this culture 0.1 ml. phage suspension (titre  $5 \times 10^{10}$  particles/ml.) was added. After incubation for 30 min. the mixture was diluted tenfold with broth and re-incubated for a further  $3\frac{1}{2}$  hr.



Prolonged incubation before testing was not essential for detection of transduction unless phenotypic expression of the new character is necessary for survival in the test *milieu*, as, for instance, in the transfer of drug resistance. It was considered desirable, however, to incubate for 4 hr., since besides giving any organisms the opportunity of expressing the new character responsible for virulence, this ensured that the cultures would be in the same phase of growth as in the virulence test.

The mixture was diluted so that 0.2 ml. contained less than one LD<sub>50</sub> and this amount was injected intraperitoneally into each of ten mice.

A measure of the efficiency of transduction in each experiment was obtained from observation of the transfer of other markers (streptomycin-resistance to strain M206 and prototrophy to strains D2 and D10) in conjunction with a viable count. Where the transfer of streptomycin-resistance was used as a marker, the phage was propagated on a streptomycin-resistant strain of the virulent culture. Two nutrient agar plates containing 500 µg. streptomycin/ml. were spread with 0.1 ml. amounts of the transduction mixture, incubated overnight, and the number of colonies counted. While lysates from a streptomycin-resistant mutant of the virulent strain were used for both mouse and *in vitro* tests, some inapparent mutation affecting virulence might have occurred simultaneously with that to streptomycin resistance, and lysates from the streptomycin-sensitive parent were also used for mouse tests. For the transduction of prototrophy the organisms from a sample of the transduction mixture were washed three times in minimal medium and made up to the original volume. Two minimal medium plates (Davis & Mingioli, 1950) were spread with 0.1 ml. amounts of the suspension, incubated for 24–36 hr., and the colonies were counted.

## RESULTS

It was found that less than 20 organisms of the virulent strains of *Salmonella typhimurium* C5 and LT2 constituted a lethal dose, the LD<sub>50</sub> doses being 14 and 17 organisms, respectively. The avirulent strains of *S. typhimurium* M206, D2, D10 and 1901 have the same virulence, the LD<sub>50</sub> doses being approximately 10<sup>7</sup> organisms. Typical results, for strains C5 and M206, are shown in Table 1.

The nature of the medium and small differences in time of incubation were not important, since growth of virulent strains of *Salmonella typhimurium* in minimal medium, serum broth or digest broth for either 4 or 6 hr. gave the same LD<sub>50</sub> dose.

Results from different experiments seldom varied, although there were differences in the time elapsing between infection and death. The majority of the mice succumbed to the virulent strains of *Salmonella typhimurium* between the 5th and the 21st days. In contrast, those deaths which occurred from the avirulent strains occurred usually within 3 days, and only an occasional animal died later. Even allowing for errors due to the small numbers of mice used at each level, the lethal doses of the virulent and avirulent strains differed by a factor of at least 10<sup>5</sup>.

Table 1. The LD 50 doses of *Salmonella typhimurium* strains C5 and M206

Organism	No. of organisms injected*	No. of mice injected†	After 30 days		LD 50 dose (no. of organisms)
			Mice living	Mice dead	
<i>S. typhimurium</i> C5	$1.2-2.5 \times 10^8$	20	1	19	14
	$1.2-2.5 \times 10^8$	25	4	21	
	$1.2-2.5 \times 10^4$	25	6	19	
	$1.2-2.5$	15	10	5	
<i>S. typhimurium</i> M206	$1.0-2.5 \times 10^7$	16	3	13	$9 \times 10^6$
	$1.0-2.5 \times 10^6$	22	18	4	
	$1.0-2.5 \times 10^5$	28	26	2	

\* The LD 50 dose was estimated on the largest number of organisms injected.

† These numbers represent the sum of separate determinations on different occasions using groups of three or five mice.

### Transductions of virulence

Three avirulent strains of *Salmonella typhimurium* (M206, D2, D10) were examined for the possible transduction of virulence. Transduction of other markers was shown to occur by the transfer of streptomycin resistance to *S. typhimurium* strain M206 and of prototrophy to strains D2 and D10. The maximum proportion of treated cells which were transformed never exceeded  $1:10^5$ .

Transduction of virulence to those avirulent strains which had an LD 50 dose of about  $10^7$  organisms was attempted, using phage propagated on organisms having an LD 50 dose of less than 20 organisms. To avoid deaths from the avirulent parent after transduction, not more than  $10^6$  avirulent organisms were injected intraperitoneally into each of ten mice. Thus only if the full virulence of the virulent strain could be transduced to a similar proportion of treated cells as was achieved for other markers—namely 1 in  $10^5$  organisms—could the experiments be expected to succeed. Repeated attempts to obtain virulent recombinants were unsuccessful and as there was no certainty that these conditions would be fulfilled it was necessary to be able to select a few virulent organisms from among many avirulent organisms. Suitable conditions for the enrichment of a few virulent organisms were ascertained from model experiments. Cultures of avirulent strains and a streptomycin-resistant variant of *Salmonella typhimurium* C5 were grown as for the virulence test. Mixtures were made, such that each mouse on injection received intraperitoneally about 10 virulent and  $10^7$  avirulent organisms, a ratio of 1:1,000,000. Two mice were killed every 24 hr. Each was skinned from the tail and the peritoneum washed out with broth in an aseptic manner. The broth was incubated overnight and changes in the ratio of virulent to avirulent organisms were ascertained by making differential counts on streptomycin-containing nutrient agar, to select the original virulent strain, and on nutrient agar to give the total viable count. The ratio gradually narrowed to 1 virulent to 9 avirulent organisms of *S. typhimurium* by the 5th day.

If the transduction of virulence is possible, this method offers the possibility of selecting a few organisms transformed to virulence. Therefore after trans-

duction a sample estimated to contain about  $10^6$  avirulent organisms was injected intraperitoneally into each of ten mice. These were killed between the 4th and 5th day, their peritoneal cavities washed out aseptically with broth and the broth washings incubated overnight. The overnight broth cultures were diluted 1/1000 in broth and incubated 6 hr. as for virulence tests. To allow rapid screening of large numbers of cultures, equal volumes of five cultures from the same transduction experiment were pooled, diluted in saline and tested for virulence. If any mice which had received less than one LD<sub>50</sub> dose of the avirulent parent died, a post-mortem examination was performed, and a pure culture of the organism recovered and retested for virulence.

Even with this technique it was not possible to select any virulent recombinants of *Salmonella typhimurium* M206, but from *S. typhimurium* D2 and D10 (both auxotrophic for adenine) having LD<sub>50</sub> doses of  $6 \times 10^6$  and  $1 \times 10^7$  organisms respectively, strains were recovered which were virulent and prototrophic. Moreover, it was found that those strains transduced to prototrophy *in vitro* and selected on minimal medium agar were also virulent. Prototrophs obtained from D2 and D10 had LD<sub>50</sub> doses of  $1 \times 10^3$  and  $4 \times 10^2$  organisms respectively, an increase in virulence of more than 1000 times that of the adenine-dependent parents.

No virulent organisms were recovered from control mice injected with the same numbers of avirulent organisms not treated with lysates of virulent strains. Thus there was no evidence of a spontaneous mutation to prototrophy *in vivo*. Prototrophic mutants were not obtained with either of these strains when  $10^{10}$  organisms were plated on minimal medium agar confirming that *in vivo* the LD<sub>50</sub> dose for mice would be reached before the appearance of prototrophic mutants might be expected.

#### DISCUSSION

Where it has been shown that hereditary characters can be transferred from one strain of organism to another, it should be possible to transfer factors responsible for virulence. An increase in mouse virulence can be transduced to *Salmonella typhimurium* strains D2 or D10, both of which are adenine-dependent (auxotrophic) strains, and the recovered organisms, besides being virulent to mice, are prototrophic. Prototrophs obtained by transduction and selection *in vitro* were also virulent, confirming that in this case the avirulence of the strain was due to a nutritional requirement which was not supplied by the tissue fluids of the mouse. A similar loss of virulence was also found in purine and in *p*-aminobenzoic acid-dependent strains of *Salmonella typhi* (Bacon, Burrows & Yates, 1951). Full virulence could be restored to these strains by reversion to prototrophy or by simultaneous injection into mice of the specific growth factor. The transduction of these strains of *S. typhimurium* to prototrophy and virulence differs, however, from the transformation to toxigenicity in *Corynebacterium diphtheriae*. In *Salmonella typhimurium* the transfer of genetic material is mediated by phage while in *Corynebacterium diphtheriae* lysogenicity is an essential prerequisite for toxin production (Freeman & Morse, 1952).



Mouse virulence has not been transduced to the avirulent *Salmonella typhimurium* M206, although streptomycin resistance can be transferred. Similarly, the adenine-dependent mutant 1901 can be transformed to streptomycin resistance but not to prototrophy; thus the strain cannot be made virulent by transduction. Phage PLT 22 propagated on strain 1901 can transduce adenine-independence to strains D 2 and D 10, which indicates that the three strains are not allelic for adenine-dependence. It seems impossible to transfer any gene which would confer adenine-independence on strain 1901. Moreover, it has not been possible to obtain back mutations to prototrophy even when as many as  $10^{11}$  organisms of strain 1901 were spread over minimal medium plates. Failure to transduce virulence to strain M206 and prototrophy to strain 1901, despite their susceptibility to transduction of streptomycin resistance, may indicate that these characters result either from mutation (or loss) of factors which cannot be transduced, e.g. a cytoplasmic particle or, more likely, from mutation of two or more factors, presumably unlinked, which cannot be transduced simultaneously by a single phage particle.

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## Flagella, Gas Vacuoles and Cell-wall Structure in *Halobacterium halobium*; an Electron Microscope Study

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**SUMMARY:** A droplet of a suspension of *Halobacterium halobium* in a concentrated brine when left to dry on a collodion-covered grid yields a useful preparation for electron microscopy. Two strains, one red and one pink, were examined. Both strains have polar tufts of flagella. The cell wall contains a single layer of hexagonally arranged globular particles. *Halobacterium halobium* has these characteristics in common with *Spirillum* spp., but unlike the true *Spirillum* spp. it is rod-shaped.

Electron microscopical examination showed that the gas vacuoles observed by Petter (1931, 1932) to be a feature of pink strains of *H. halobium* actually were smaller but much more numerous than the optical microscope revealed. Stereopictures show that these vacuoles did not collapse upon desiccation of the bacteria.

Red discoloration of salted fish can be caused by at least three distinct species of bacteria. In most cases a rod-shaped bacterium has been isolated from the substrate. *Bacillus halobius ruber* is the name Klebahn (1919) gave to a red rod-shaped halophilic bacterium. Petter (1931) changed the name to *Bacterium halobium* because it does not form spores. According to her, *Pseudomonas salinaria* Harrison et Kennedy is the same species. More recently, Elazari-Volcani (1940) attached the generic name *Halobacterium* to it, *H. halobium* being the type species.

In 1954, Mr de Melker, working in the Laboratory for Microbiology at Delft, obtained some strains of this species. Originally the colonies of all the strains were transparent and red. Some of the strains, however, on being subcultured for some time on proteose peptone NaCl (23 %, w/v) agar, began to make opaque pink colonies. This was what Petter had described in her preliminary paper (1931) and in her thesis (1932). In other respects also Mr de Melker's strains did not seem to differ from those which Petter had examined. Petter made a thorough investigation of the characteristics of several strains of the bacteria of salted fish. She found that the red and the pink strains of the rod-shaped species had in common a mixture of two closely related carotenoid pigments. The macroscopically different appearance of cultures of the two strains was paralleled by a different appearance under the microscope: the pink organisms contained a variable number of 'bodies' with a low refractive index while the red organisms did not. The results of several experiments led Petter to the conclusion that these 'bodies' in the pink bacteria consisted of, or contained, gas. (Compare the phase-contrast micrographs, Pl. 1, figs. 1, 2). The main argument was, and still is, that these 'bodies' can be made to disappear by subjecting a suspension of pink bacteria to a pressure of a few atmospheres. The result is immediately visible: the suspension turns red.

Microscopical examination shows that no 'bodies' are left, so the opacity as well as the different colour is clearly due to the presence of these 'bodies'. The latter Petter named 'gas vacuoles' as she considered them comparable to the gas vacuoles which Klebahn had described in Cyanophyceae. Though Petter collected other evidence that the 'bodies' in her bacteria were indeed gas vacuoles, some later authors have disagreed with this interpretation. The fact that different authors have used different strains may partly explain the contrary views. Spruit & Pijper (1952) examined a strain having a morphological form which showed many twisted cells. A comparison of the image observed in their microscope with the micrographs on p. 45 of Petter's thesis made them apply to the appearance of Petter's organisms an interpretation of their own, namely, that the represented bacteria were twisted organisms and not organisms containing gas vacuoles. Spruit & Pijper's interpretation might have differed less from Petter's, had they studied the latter author's strain directly, focusing up and down on the organisms as she did. According to a later paragraph in Spruit & Pijper's article, not all of the images observed by Petter should be explained by assuming all the organisms to be twisted; some might contain highly light-scattering particles. To call these bodies 'products of degeneration' does not, however, discharge the authors of the duty of explaining the unusual physical properties which led Petter to infer that these 'bodies' were gas vacuoles.

#### METHODS

Because of the presence of the gas vacuoles, the specific weight of bacteria of the pink strain is less than that of a concentrated brine (e.g. 23 %, w/v, NaCl). When a suspension of these bacteria in 23 % NaCl solution is centrifuged, a pink layer forms on top of the supernatant fluid. However, only organisms originally near the surface gather in this layer. The other organisms are subjected to so much pressure that this causes the gas vacuoles to collapse. Therefore centrifugation also yields a red pellet, and is not a suitable method to use when pink bacteria are to be prepared for electron microscopical examination.

For another reason the usual procedure of washing the organisms with distilled water before desiccation on the collodion membrane cannot serve in this case. The organisms turn into a slimy mass as soon as the medium is diluted with water. If this bacterium could be adapted to a medium containing only a little NaCl, such organisms might perhaps survive suspension in distilled water. It has been found, however, that a medium with less than *c.* 12 % (w/v) NaCl does not support growth.

Good results were obtained with organisms taken directly from the pink layer floating on top of an undisturbed liquid medium. Small drops of this layer were diluted with a 27 % (w/v) NaCl solution; a droplet of the suspension was placed on top of a collodion-covered grid and partly sucked off with filter-paper. It now proved an advantage that these bacteria were adapted to a concentrated brine: during desiccation the cells found themselves in natural surroundings up to the last moment.



After desiccation the specimens were shadowed with platinum. Electron micrographs were taken with a modified Philips E.M. 100 instrument, which has a short focal-length objective lens designed by Le Poole. Although most of the organisms were to a greater or lesser extent obscured by small NaCl crystals, a small percentage of organisms escaped being hidden in this way and electron micrographs were made from these. Stereopictures were taken by tilting the object by *c.* 7 degrees to the right and as much to the left, alternately.

For the present investigation one red and one pink strain were selected from the strains of Mr de Melker.

## RESULTS

### *Flagella*

A high percentage of the organisms showed motility, though the movements were restricted to axial rotation and oscillation of the poles. Some micrographs (see Pl. 1, figs. 3, 4) show bacteria with polar tufts of flagella. This is in agreement with the findings of Harrison & Kennedy (1922) and of Lochhead (1934) who succeeded in staining the flagella. Though other authors failed in an attempt to make these organelles visible, none but Spruit & Pijper (1952) have claimed that this apparently motile bacterium lacks flagella.

### *Gas vacuoles present in the pink strain*

In electron micrographs of bacteria, e.g. *Mycobacterium tuberculosis* (Knaysi, Hillier & Fabricant, 1950; Brieger, Cosslett & Glauert, 1954; Scanga, 1954) and *Caulobacter vibrioides* (Bowers, Weaver, Grula & Edwards, 1954) ordinary vacuoles are known to show up as less dense areas. In the present investigation the same was found with the gas vacuoles. There were, however, some differences. Ordinary vacuoles become quite flat upon desiccation. The adjacent cytoplasm contains a lower percentage of water and therefore flattens less. Micrographs of shadowed specimens show that the less dense areas are simply the flattest areas of the bacterial body; the upper cell wall has sunk down to the bottom of what looks a shallow pit and which is the site of the collapsed vacuole.

The vacuoles of the pink bacteria, as shown in Pl. 4, fig. 1, apparently do not collapse; the site of a vacuole is hardly flatter than the adjacent cytoplasm.

Stereopictures (Pls. 2, 3) give a much better insight into the three-dimensional structure of the desiccated bacteria. On original prints the observer sees the upper cell wall as nearest, and the gas vacuoles in various planes, in some places one behind the other.

The original volume of the gas vacuoles will not necessarily have been decreased by desiccation. Their shape, on the other hand, may have altered. The cytoplasm has lost water, and therefore the whole of the bacterial body, gas vacuoles included, must have flattened. At the same time the cytoplasm around the gas vacuoles will have contracted also in the horizontal plane. Both causes will have co-operated in making the gas vacuoles lens-shaped instead of spherical, and therefore in making them look very large. Unfortunately this results in the micrographs falsely suggesting the organism to be

almost filled with gas vacuoles, and the latter to be separated from one another by little more than a membrane. This appearance may cause objections to be raised against the method used in preparing the specimens. One may doubt, however, whether any other method of dehydration would result in a truer representation of the bacterium.

The distances between many of the gas vacuoles must be rather small since the optical microscope appears unable to show all of them as separated. The electron microscope shows how large their number really is.

The lesser density with respect to the medium, as shown by the ability of the pink organisms to float on top of it, is but one consequence of the presence of gas vacuoles. Since the gas vacuoles fill part of the space within the cell wall, another consequence is that the ratio 'volume of the cytoplasm/surface area of the cell wall' is smaller than it would be if the gas vacuoles were lacking. This ratio is also determined by the shape of the organism. In ribbon-shaped organisms, for example, the ratio is smaller than in cylindrical ones. In this connexion it is interesting that Spruit & Pijper (1952), studying a red strain of *Halobacterium halobium* which lacks gas vacuoles, found 'that the majority of the individuals did not have the usual cylindrical form of bacteria, but were more or less flat, bent, or curled ribbons'. Also in the present investigation flat organisms were found in great numbers in cultures of the rod-shaped halophilic bacterium, especially with the red strain. Therefore the red strain of *H. halobium* has, in common with the pink, a smaller ratio 'volume of the cytoplasm/surface area of the cell wall' than would be found if the organisms were cylindrical and if they lacked vacuoles. In growing organisms the same must be true of the ratio 'newly formed cytoplasm/newly formed cell wall material'; it follows that both in vacuolated and in flat organisms the proportion of assimilated nutrients turned into cell-wall material is comparatively large. It is quite another question whether a large surface area with respect to the volume of the cytoplasm is profitable to the organism. There was found no indication that this was the case with *H. halobium*.

### *The cell wall*

Some of the micrographs (Pls. 2, 4) of the pink organisms, as well as some of the red organisms, show a cell-wall structure very similar to that of *Spirillum* spp. (Houwink, 1953). Globular particles about 130 Å. wide are arranged in one layer in a hexagonal pattern. If the globular particles in the bacterial cell wall are macromolecules—and this does not seem too wild a speculation—this layer of the cell wall may be a two-dimensional crystal lattice. Since a crystal grows by apposition only, and not by intussusception, this cell wall will have definite growth zones, coincident with the borders of the crystalline areas. Such borders must occur at the poles of the cell, where the shape of the cell wall passes from the tube into the dome. Additional growth zones may exist in other parts of the cell wall, wherever a dislocation occurs in the regular pattern of the globules. A similar cell-wall structure has not been found in any *Pseudomonas* sp. thus far. This may be one reason to consider *Halobacterium*

*halobium* more closely related to the genus *Spirillum* than to the genus *Pseudomonas*.

The author wishes to thank Professor A. J. Kluyver for his interest in this work and for his valuable criticism.

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#### EXPLANATION OF PLATES

##### PLATE 1

- Fig. 1. Phase-contrast micrograph of *Halobacterium halobium*, red strain. Magnification,  $\times 1500$ .
- Fig. 2. Phase-contrast micrograph of *H. halobium*, pink strain. Magnification,  $\times 1500$ .
- Figs. 3, 4. Electron micrographs of *H. halobium*, pink strain. Tufts of flagella. Magnification,  $\times c. 35,000$ .

##### PLATE 2

Stereo electron micrograph of *H. halobium*, pink strain. Magnification,  $\times c. 60,000$ .

##### PLATE 3

Stereo electron micrograph of *H. halobium*, pink strain. Magnification,  $\times c. 52,500$ .

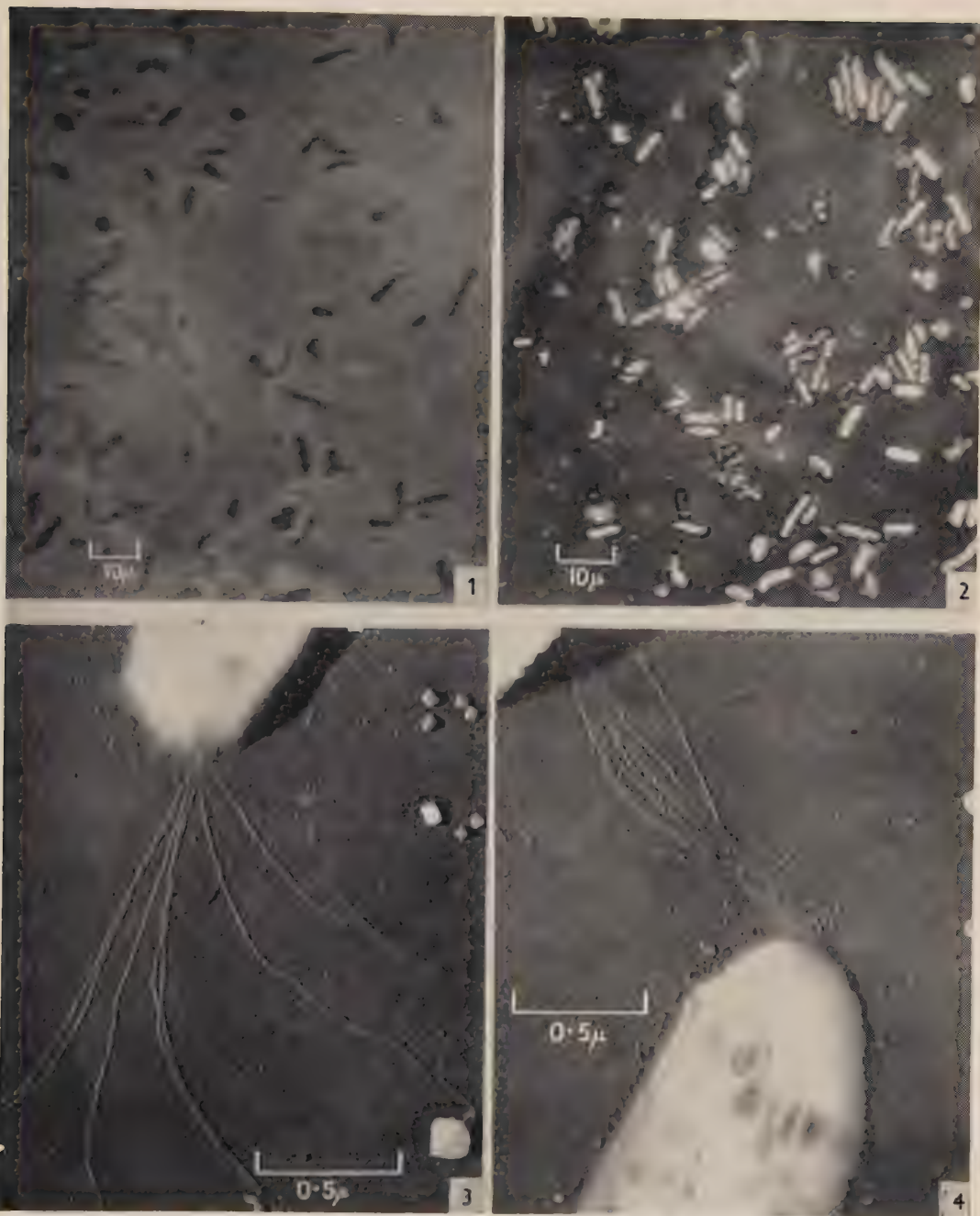
##### PLATE 4

- Fig. 1. Electron micrograph of *H. halobium*, pink strain. Magnification,  $\times c. 60,000$ .
- Fig. 2. Electron micrograph of *H. halobium*, red strain. Magnification,  $\times c. 60,000$ .

A simple stereoscope may be made of two identical magnifying glasses with a diameter of c. 6 cm. and a focal length of c. 20 cm. The distance between the glasses should be adjustable to the observer's need.

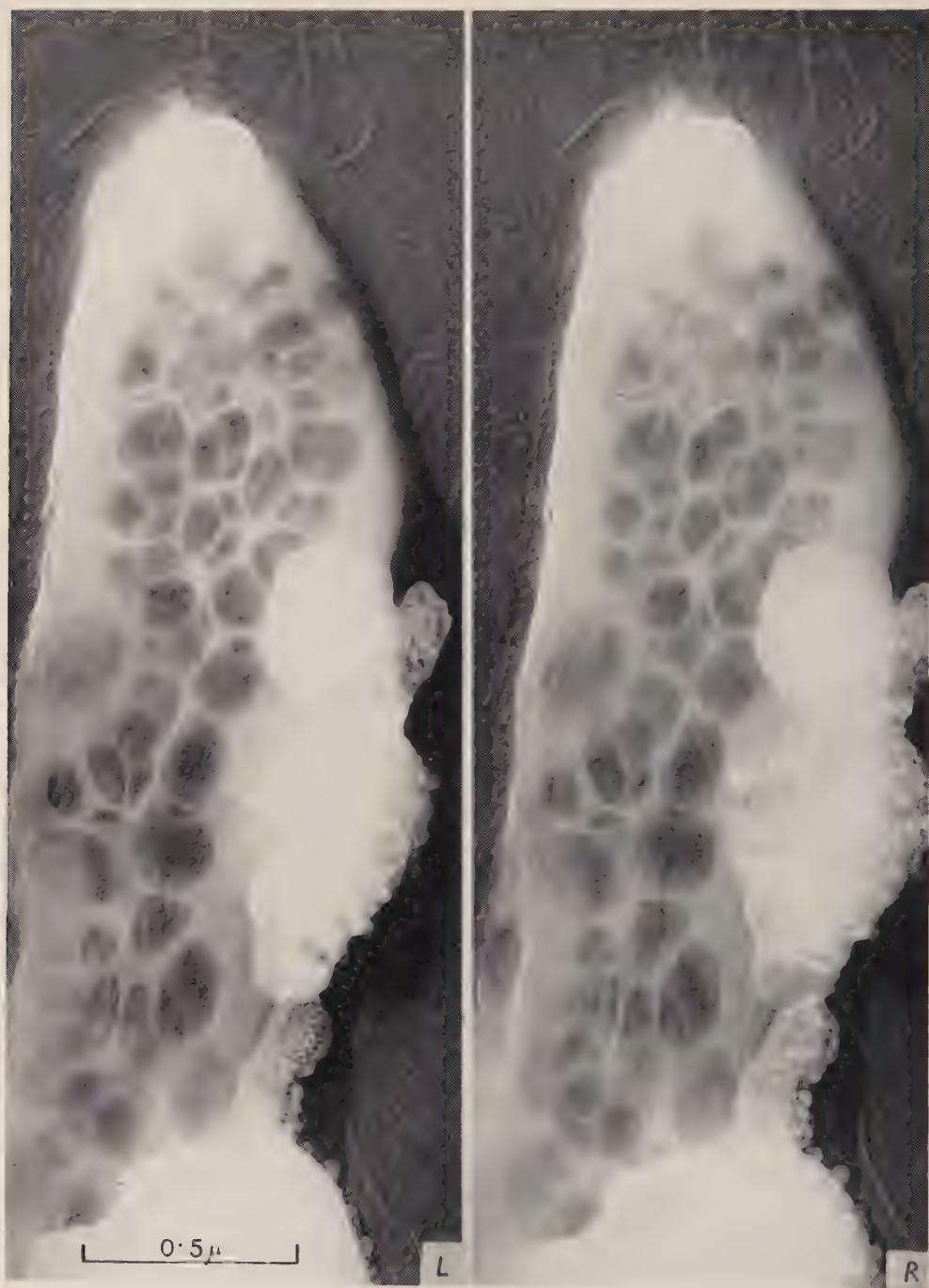
(Received 23 January 1956)



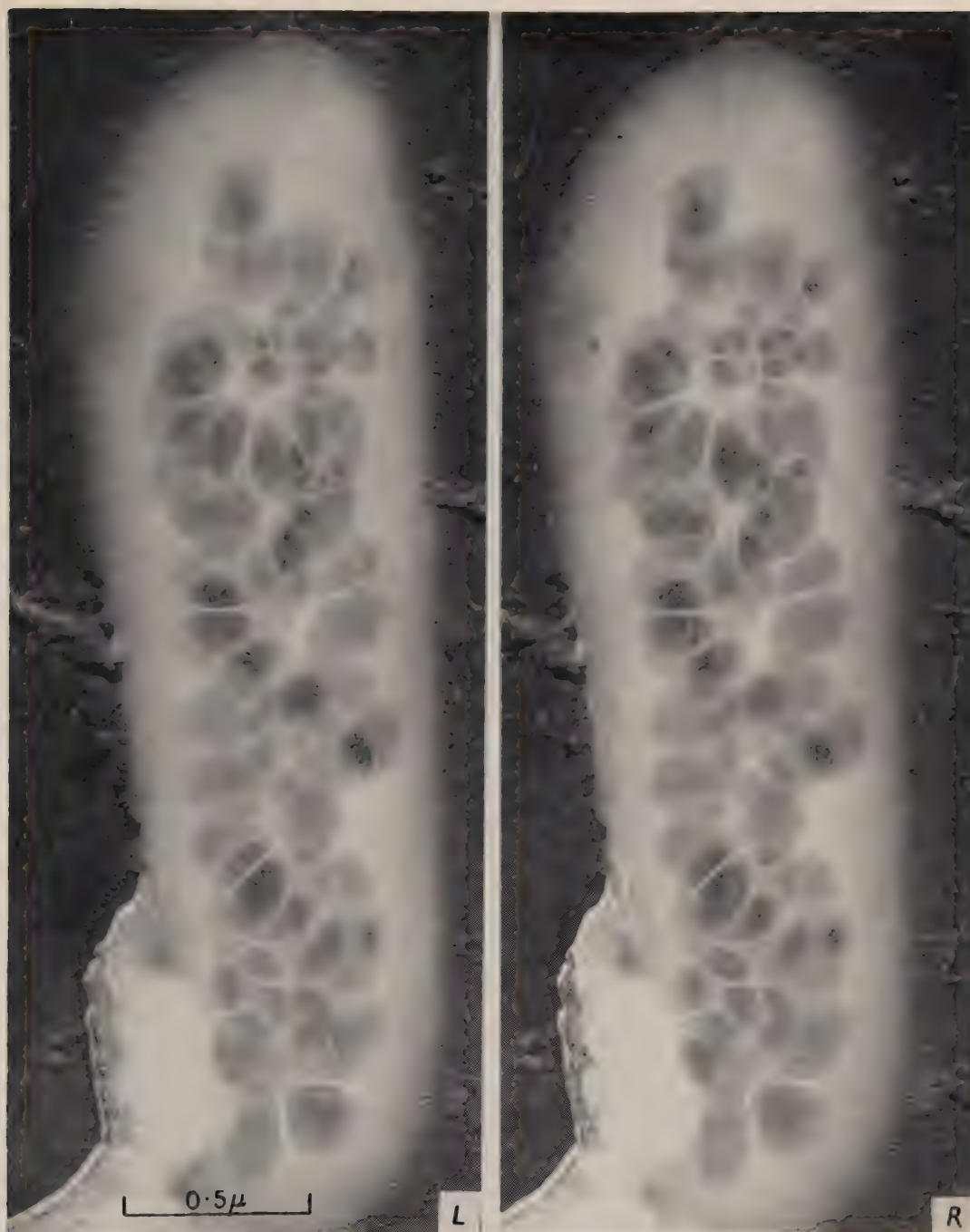


A. L. HOUWINK—GAS VACUOLES AND CELL WALL IN *HALOBACTERIUM*. PLATE 1

(Facing p. 150)



A. L. HOUWINK—GAS VACUOLES AND CELL WALL IN *HALOBACTERIUM*, PLATE 2



A. L. HOUWINK—GAS VACUOLES AND CELL WALL IN *HALOBACTERIUM*. PLATE 3





A. L. HOUWINK—GAS VACUOLES AND CELL WALL IN *HALOBACTERIUM*. PLATE 4

UDEN, N. VAN, MATOS FAIA, M. DE & ASSIS-LOPES, L. (1956). *J. gen. Microbiol.* 15, 151-153

## Isolation of *Candida albicans* from Vegetable Sources

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**SUMMARY:** Five strains of *Candida albicans* were isolated from the following vegetable sources: flowers of *Ulex* sp. (two strains), leaves of *Myrtus communis*, a sample of 'hongo' and a sample of sour red wine. The five strains show the characteristic properties of *Candida albicans* including pathogenicity for rabbits. These isolations do not prove that the organism has a saprophytic free-living stage; only that it can at least survive outside an animal host for an appreciable time.

The yeast *Candida albicans* has its known habitat in the mouth, throat, intestinal tract, and perhaps other inner body surfaces of man, certain mammals and birds, and it can readily be isolated from many healthy individuals. Under special and only partly recognized conditions (diabetes, pregnancy, prostrating diseases, antibiotic treatment) this yeast may cause disease ('moniliasis') of a variety of organs (mouth, intestine, vagina, skin, bronchi, lungs, avian crop). As *C. albicans* has been isolated almost exclusively from man and animals, it is generally assumed that infection with this yeast takes place from man or animal to man or animal by intimate contact. *C. albicans* has until now been reported as isolated only twice from non-animal sources. According to Skinner (1947) *C. aldoi* (now considered a synonym of *C. albicans*) was isolated by Negroni and Fisher from decaying vegetables; and di Menna & Parle (1954) and di Menna (1955) isolated three strains of *C. albicans* from two soils in New Zealand.

Among 180 yeast strains which we isolated from plants, vegetable- and dairy-products, five strains belonged to *Candida albicans*; two were isolated from flowers of furze (*Ulex* sp.), one from leaves of myrtle (*Myrtus communis*), one from a sample of 'hongo' (a symbiosis of *Acetobacter xylinum* with various yeasts used for preparing a popular beverage from sugared tea) and one from sour non-bottled red wine. The two furze plants were located near the top of a hill (300 m. high) near Vermoil in the Portuguese province of Estremadura. The approximate height of the flowers on the bushes was 1 m. above the ground; the hill was uncultivated and uninhabited by man, but occasionally a small herd of sheep and goats used to pass. The myrtle grew on the slope of the same hill and the leaves from which *Candida albicans* was isolated were c. 0.5 m. above the ground. The sample of 'hongo' was obtained in Lisbon from a man who had handled this material during several months in passing it every 3 or 4 days to a fresh lot of sugared tea. The wine was purchased in a small shop in Lisbon.

The five strains form round and slightly oval cells on solid and liquid media. None of the strains formed ascospores when grown on various sporulation media (gypsum blocks, Gorodkowa agar, carrot plugs). On Dalmau plates (Wickerham, 1951) of glucose (2%) yeast extract (0.5%) peptone (1%) agar the formation of pseudomycelium with ball-like clusters of blastospores was observed in all five strains. Besides this type of pseudomycelium, other types as described by Lodder & Kreger-van Rij (1952) for *Candida albicans* were seen in various degrees. The present authors did not feel it necessary for the identification of the strains to try to induce the formation of chlamydospores. The fermentation and assimilation tests were done according to Wickerham's (1951) technique with the following results: D-glucose and maltose strongly fermented, D-galactose weakly fermented; sucrose and lactose not fermented. The strains utilized: D-glucose, D-galactose, sucrose, maltose, D-xylose, ethanol, D-mannitol, D-sorbitol and soluble starch; L-arabinose utilized very weakly; nitrate, lactose, raffinose, inositol, inulin are not utilized.

Intravenous inoculation of rabbits with 1 ml. of a 1% (v/v) suspension of organisms of any of the strains resulted in death of the animals within 4 days with the formation of numerous abscesses in the renal cortex and the brain.

Our 5 strains could be differentiated from *Candida stellatoidea* and *C. clausenii*, two species closely related with *C. albicans*. *C. stellatoidea* does not assimilate sucrose and does not kill rabbits when 1 ml. of a 1% (v/v) suspension of organisms is inoculated intravenously. *C. clausenii* does not ferment galactose, is morphologically distinct from *C. albicans*, and is less pathogenic for laboratory animals.

Our findings do not prove that a natural habitat of *Candida albicans* is on plants and in various vegetable products. It seems more probable that non-animal substrates like ours may support growth or permit survival of *C. albicans* during considerable time, after being contaminated with the yeast from man or animals. In the case of the wine and the 'hongo' the original infection almost certainly was from man. The plants might have been contaminated by animals (e.g. sheep, goats, birds, rodents, man). As for the time of survival of *C. albicans* on non-animal substrates, some evidence was obtained by di Menna & Parle (1954); they isolated *C. albicans* during 9 months from artificially inoculated turf. Our findings show that various vegetable materials may harbour *C. albicans*, and it is evident that this yeast could be introduced into man or animals on ingestion of those materials.

Three of the five strains we isolated are maintained in the culture collection of the Departamento de Micologia, Instituto Botânico, Lisbon, under the following numbers: 2427 (from *Myrtus communis*), 2428 (from *Ulex* sp.), 2491 (from 'hongo'). Cultures of the same strains have been deposited with the Yeast Division of the Centraalbureau voor Schimmelcultures, Delft, Holland.

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## The Cell-bound Penicillinase of *Bacillus cereus*

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**SUMMARY:** Only 30-50 % of the cell-bound penicillinase of *Bacillus cereus* NRRL 569 is neutralized by antiserum prepared against the exo-penicillinase. The un-neutralizable fraction is not decreased by cell disintegration which liberates a proportion of the cell-bound enzyme into solution. Absence of neutralization cannot therefore be explained by the existence of a mechanical barrier which prevents access of antibody; it has in fact been shown to be due to the presence of another type of penicillinase, not liberated from the cells, which is enzymically similar to, but immunologically and physico-chemically distinct from, the exo-enzyme. This cell-bound penicillinase has been separated from the exo-enzyme by fractional precipitation with ammonium sulphate. It is inducible by penicillin, like the exo-enzyme, and is present in cells of the constitutive mutant strain, 569/H.

The penicillinase of *Bacillus cereus* strain 569, like that of other strains of this species, is mainly exocellular. With liquid cultures in broth or casein hydrolysate only about 10 % of the total activity is found attached to the organisms after centrifugation. This proportion has not been found to vary markedly with conditions of culture, phase of growth or extent to which total penicillinase production is increased by induction with penicillin (see Pollock, 1952). It was thought that some information might be obtained on the localization of this cell-bound enzyme by studying the action of an anti-penicillinase serum. Krebs & Wright (1951) found that an antiserum to purified yeast triosephosphate dehydrogenase caused 90 % inhibition of activity of the isolated enzyme, although it had no effect on glucose fermentation in intact organisms; they concluded that antibody molecules could not penetrate the cell wall. Analogous findings were reported by Pasternak, Sevag & Miller (1951) with yeast hexokinase and carboxylase, neither of which was affected in intact organisms by treatment with antisera which neutralized the enzymes after extraction from the organisms. Sevag, Newcomb & Miller (1954) discovered that the cell-bound  $\alpha$ -glycerophosphatase of dried yeast could be completely inhibited by a specific anti- $\alpha$ -glycerophosphatase serum and so concluded that the enzyme must be 'at or near the cell surface'. It seems to be a fair assumption that antibody molecules of  $\gamma$ -globulin cannot penetrate inside normal, intact microbial cells. Specific neutralization of the activity of cell-bound enzyme by an antiserum can therefore reasonably be taken as an indication that the enzyme is on the cell surface. In the work reported here it was found that only about 50 % of the cell-bound enzyme activity could be neutralized by antiserum which neutralized nearly completely the activity of the exo-enzyme (Pollock, 1956). Attempts to understand the reasons for this absence of neutralization led to the discovery of an immunologically and physico-chemically distinct type of penicillinase which was probably situated inside the cell wall.

## METHODS

*Organism and enzyme formation.* *Bacillus cereus*, NRRL 569, was grown from a spore inoculum in 'S' peptone broth (Pollock & Perret, 1951) containing 1% (w/v) gelatin (to prevent enzyme inactivation), shaken at 35°. Without addition of penicillin only small amounts of enzyme were produced. In order to induce the formation of penicillinase, 1 unit benzylpenicillin/ml. was added at a concentration of organisms corresponding to 0.1 mg. dry weight organisms/ml. and incubation continued for up to 60 min. Oxine (8-hydroxyquinoline) at a concentration of  $8.3 \times 10^{-4}$  M was added to stop further enzyme formation, and all enzyme preparations (whether of soluble exo-enzyme, suspensions of whole organisms or disrupted organisms and extracts therefrom) were prepared, unless stated otherwise, in the presence of  $8.3 \times 10^{-4}$  M-oxine and 1% gelatin.

The mutant strain 569/H of *Bacillus cereus*, which produces penicillinase constitutively (subsequently referred to as being of a 'constitutive-penicillinase' type) was derived from strain 569 (see Kogut, Pollock & Tridgell, 1956). It was used for a few experiments and was grown in a similar manner, except that no additions of penicillin were necessary for maximal production of penicillinase.

*Antiserum.* A solution of  $\gamma$ -globulin was prepared from the serum of a rabbit immunized with purified induced exo-penicillinase from *Bacillus cereus*, strain 569 (Pollock, 1956).

*Enzyme assay.* Total enzyme was normally assayed manometrically at pH 7.0 by the method of Henry & Housewright (1947) using 1.0 ml. samples in oxine and gelatin. The 'unneutralizable' moiety is expressed as a percentage of the total activity remaining after addition of sufficient antibody to neutralize four equivalents of exo-enzyme. Now, when anti-penicillinase serum is added to exo-enzyme, there always remains from 2 to 4% of the activity unneutralized, even in gross excess of antibody. This was shown to be due to the residual enzymic activity of the enzyme-antibody complex (Pollock, 1956) and has nothing to do with the present problem except in so far as it decreases the accuracy of assay of the 'unneutralizable fraction' in a mixture. When the latter forms a low proportion of total activity, there may be considerable error in its determination. But the error becomes progressively less as the proportion of unneutralizable fraction rises ( $> 8\%$  in a mixture of equal parts) and does not significantly affect the results reported here. An example may illustrate the technique used. Suppose a suspension of organisms or disintegrated cell debris is found to have a total enzyme activity of 25 units/ml. A sample (usually 2.0 ml.) is mixed with 0.5 ml. antiserum  $\gamma$ -globulin containing a total of 100 569-neutralization units of antibody. The mixture is assayed after 30 min. at room temperature and the activity expressed as a percentage of that of the untreated sample. The neutralization titre of the antibody preparation is measured by accurate assay of the activity which remains after mixing a constant amount of the 569 exo-enzyme with varying quantities of antiserum (Pollock, 1956), one neutralization unit being defined



as that amount of antibody which will neutralize 1 unit of penicillinase in the presence of excess enzyme.

For the determination of penicillinase activity at different pH values, the iodometric assay technique of Perret (1954) was used. For the determination of Michaelis constants, enzymic activity was measured by cup-plate bio-assay of the residual penicillin, with *Bacillus subtilis* I.C.I. strain as test organism (Humphrey & Lightbown, 1952) since the affinity of penicillinase for its substrate is so high that manometric assay of the reaction at concentrations of penicillin insufficient to saturate the enzyme is impossible. It was found that at an enzyme concentration of about 0.02 unit/ml., penicillin could be accurately assayed in the presence of enzyme provided that: (a) the reaction mixture was diluted at least tenfold in ice-cold phosphate buffer before sampling in the cups; (b) the penicillin in the cups was allowed to diffuse into the surrounding agar of the assay plate at 2° for at least 4 hr. before incubation. In this way reaction velocities could be measured by incubation for 60 min. at 30° in 1% gelatin with 0.01 M-phosphate (pH 7.0) at penicillin concentrations down to 10 units/ml., taking samples every 15 min. All penicillin samples were assayed in eight replicates. The maximum enzyme activity measured by this technique was found to be about 15% higher than that measured manometrically.

## RESULTS

Fig. 1 shows the effect of adding increasing amounts of anti-penicillinase antibody to: (a) exo-enzyme (penicillin-induced culture supernatant fluid); (b) organisms from the same culture centrifuged and resuspended in oxine + gelatin; (c) organisms washed three times in oxine + gelatin; (d) organisms washed five times before resuspension. It can be seen that about 40% of the cell-bound enzyme in (b) was neutralized by antibody, and that most of this neutralizable, bound enzyme was only loosely bound to the organisms since it was removed by repeated washing, leaving the unneutralizable moiety still attached (d). The neutralization slope of that fraction of the cell-bound enzyme, which was easily removed by washing the organisms, was similar to that of the exo-enzyme, and shows that these two fractions of enzyme are immunologically identical or very closely related. Indeed, the process of washing off, which will occur at +2° in the presence of oxine, appears to be rather in the nature of mechanical removal, from absorption on the surface of the organisms, of 'mature' enzyme, otherwise indistinguishable from exo-enzyme. Addition of organisms from an uninduced culture had no effect on the neutralization by antibody of exo-enzyme (induced culture supernatant) and thus confirmed that the unneutralized cell-bound fraction was a distinct moiety which was for some reason unable to react with antibody. It seemed at first natural to attribute this absence of neutralization to the presence of some barrier, associated with intact cell structure, which allowed penicillin to reach the enzyme but prevented access of the relatively large molecules of immune rabbit  $\gamma$ -globulin.

However, disintegration of the organisms did not lead to increased neutrali-

zation of enzyme by antiserum. Table 1 summarizes the results of four experiments with previously induced cultures of *Bacillus cereus*, strain 569, in which 7 ml. of a thick suspension of washed organisms were crushed in the Hughes press at  $-30^{\circ}$ , in the presence of  $8.3 \times 10^{-4}$  M-oxine and 1% (w/v) gelatin. Microscopic examination of cell material after such crushing revealed a mass of debris and partially disintegrated cells but no significant proportion of normal intact organisms. The total penicillinase activity after disintegration (A) was on an average about 8% less, and in no instance greater than that of unbroken

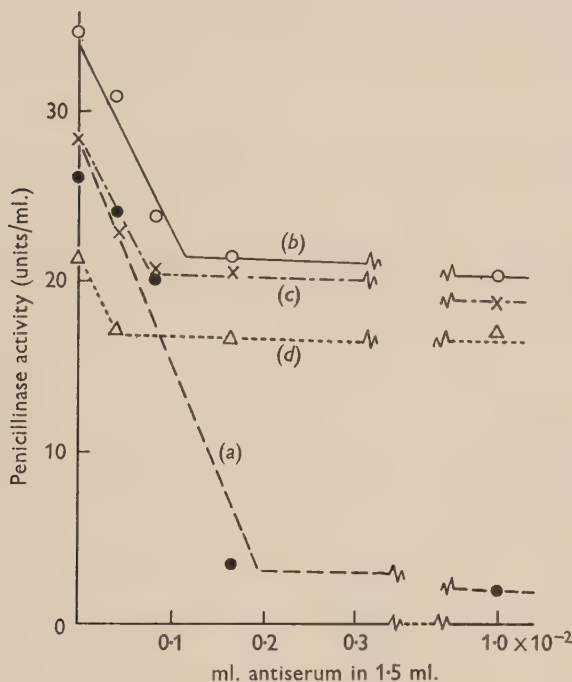


Fig. 1. Effect of antibody (prepared against exo-penicillinase) on penicillinase activity of supernatant fluid and cells of a penicillin-induced culture of *Bacillus cereus* 569. ---●---●---, supernatant fluid (=exo-penicillinase); —○—○—, unwashed organisms, resuspended after centrifugation; ---×---×---, 3 times washed organisms; ...△...△..., 5 times washed organisms.

organisms, so confirming that the intact cell structure offered no barrier to access of penicillin to the enzyme. Moreover, the preparation of disintegrated organisms showed the same proportion of unneutralizable activity as the intact organisms. The disintegrated organisms were then suspended in an equal volume of oxine+gelatin mixture and divided into two portions: one was centrifuged for 15 min. at 2000 *g* as soon as the ice crystals had melted, and the supernatant fluid (B) separated; the other portion was incubated at  $35^{\circ}$  for 10 min., to allow the mass to melt and the whole preparation to be homogenized by stirring, and was then centrifuged and the supernatant fluid (C) separated. It can be seen that the supernatant B contained on an average

about 25 % of the activity extracted in supernatant C, but the percentage of unneutralizable enzyme was in some instances disproportionately high. Altogether about 40 % of the total cell-bound activity and 20 % of the unneutralizable cell-bound activity was thus eventually liberated into solution, but the total amount of unneutralizable activity was never increased. Thus although a proportion of the cell-bound enzyme appeared to be 'intracellular' (in the sense that it was not detached from the organisms until they were broken), its inability to be neutralized by antiserum could not be explained by the presence of some solid barrier preventing access of antibody molecules. It seemed reasonable to suppose that a proportion, at least, of the unneutralizable penicillinase moiety consisted of some immunologically distinct type of molecule or molecular complex.

Table 1. *Total and unneutralizable penicillinase activities found in washed penicillin-induced Bacillus cereus 569 before and after disruption in the Hughes press and subsequent extraction into solution*

For full details see text. Results are expressed as: (a) total penicillinase activities in units per mg. dry weight of the bacterial cells from which the preparation was derived; (b) as percentage of this activity which is not neutralized by an antiserum to the exo-enzyme.

Expt.	Period of incubation after induction with penicillin (1 unit/ml.) (min.)	Soluble fraction from disrupted organisms							
		Intact organisms		(A) Disrupted organisms (total)		(B) Rapid extraction		(C) Extraction after incubation at 35° for 10 min.	
		(b) Unneutralizable		(b) Unneutralizable		(b) Unneutralizable		(b) Unneutralizable	
		(a) Total	(%)	(a) Total	(%)	(a) Total	(%)	(a) Total	(%)
1	40	—	—	5.22	52	0.54	94	2.10	51
2	40	7.15	58	6.80	57	1.47	79	—	—
3	60	17.1	66	15.6	65	2.21	71	7.95	54
4	75	20.5	72	18.5	69	2.54	64	9.38	51

For convenience, the penicillinase of *Bacillus cereus* has been divided empirically into different fractions based primarily on their ability to be neutralized by an antiserum prepared against the exo-enzyme and the degree to which they are associated with sedimentable solid cell material, as shown in Table 2. The  $\alpha$ -fraction is simply the 'normal' exo-enzyme, liberated into the culture medium. It has been purified, and characterized in some detail (Kogut *et al.* 1956). The  $\beta$ -fraction, most of which can be removed from the organisms by repeated washing, is the neutralizable moiety of the cell-bound enzyme. It is immunologically indistinguishable from the  $\alpha$ -fraction. The  $\gamma$ -fraction is that portion of the unneutralizable cell-bound activity which can be extracted into solution after disintegration of the organisms. The relative activities of the various fractions are indicated because they were found to be approximately constant in several experiments with induced cultures and did not differ markedly from those obtained before induction.



Table 2. *Distribution and properties of different penicillinase fractions obtained from penicillin-induced Bacillus cereus 569*

	Penicillinase activity (% of total)	Descriptive term
Exo-cellular	85-95	$\alpha$ -Penicillinase
Cell-bound:		
(a) Total	5-15	'Cell-bound' enzyme
(b) Neutralized by antiserum to exo-enzyme	1-8	$\beta$ -Penicillinase
(c) Not neutralized by antiserum to exo-enzyme		
(i) Total	4-7	'Unneutralizable' fraction (normally all $\gamma$ -penicillinase)
(ii) Extractable into solution	2-3	$\gamma$ -Fraction ( $\gamma$ -penicillinase)

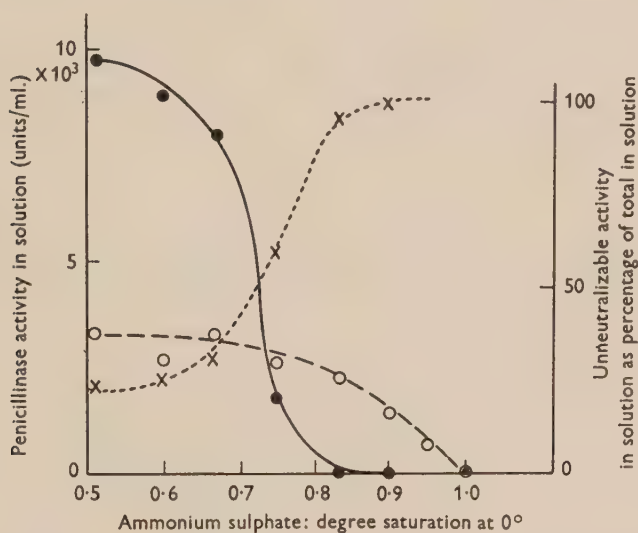


Fig. 2. Separation of  $\gamma$ -penicillinase from the neutralizable cell-bound penicillinase ( $\beta$ -fraction) by fractionation, in ammonium sulphate, of a preparation from an autolysate of washed penicillin-induced *Bacillus cereus*, 569. Plot of activities remaining in supernatant fluid after centrifuging off precipitate at different concentrations of ammonium sulphate. —●—●—, penicillinase activity neutralized by antiserum to exo-enzyme; —○—○—, penicillinase activity not neutralized by antiserum; ... x ... x ..., percentage of total activity not neutralized by antiserum.

*Isolation of  $\gamma$ -penicillinase.* Approximately 500 g. dry weight of *Bacillus cereus*, strain 569, from 320 l. induced culture grown in casein hydrolysate and used for preparation of purified induced-penicillinase (Kogut *et al.* 1956) was washed with 2 l. of  $8.3 \times 10^{-4}$  M-oxine in 0.01 M-phosphate (pH 7.0), resuspended in 2 l. oxine + phosphate and incubated for 16 hr. at 35°. This treatment allowed considerable autolysis of the organisms to occur. The cell debris was centrifuged down as far as possible and the supernatant fluid, containing a total of  $2.5 \times 10^6$  units penicillinase (of which 23% was unneutralizable), was saturated with ammonium sulphate and the precipitate allowed to flocculate at 2° overnight.

The massive precipitate was filtered off, suspended in 100 ml. water and dialysed against  $5 \times 10^{-3}$  M-phosphate (pH 7.0). Most of the precipitate dissolved and, after spinning off insoluble matter, the supernatant fluid (150 ml.) was dialysed successively at 2° against increasing concentrations of ammonium sulphate at pH 6.0, starting at 0.50 saturation. After separating the precipitate that was formed at each concentration and assaying both the supernatant fluid and precipitate (dissolved in 0.1 M-phosphate; pH 7.0) for total and unneutralizable penicillinase activity, the supernatant fluid was dialysed against the next higher concentration of ammonium sulphate. This fractional precipitation was continued until a concentration of 0.90 saturated ammonium sulphate had been reached, at which point the enzyme remaining in solution was 99% unneutralizable by antiserum (see Fig. 2). The yield of  $\gamma$ -fraction, thus freed from the  $\beta$ -fraction, was only just over 5% of that present in the extract from autolysed organisms, but was enough to allow some investigation of its properties. The fact that it was possible to separate it almost completely from the 'normal' enzyme by a simple fractionation procedure, based on differences in solubility in ammonium sulphate, showed that it belonged to a distinct molecular kind of protein, which is henceforth referred to as ' $\gamma$ -penicillinase'.

#### *Properties of $\gamma$ -penicillinase*

The solution of  $\gamma$ -penicillinase so obtained was free from  $\alpha$ - and  $\beta$ -fractions, but was likely to be contaminated with other proteins. Moreover, only very small quantities (about  $5 \times 10^4$  units) were available so that direct investigation of its physico-chemical properties was not attempted.

Table 3. *Relative rates of hydrolysis of different penicillins by  $\gamma$ -penicillinase and exo-penicillinase of Bacillus cereus 569*

Enzyme	Relative activity on substrates (benzylpenicillin = 100)		
	Penicillin K	Cephalosporin N	Phenoxymethyl penicillin
Exo-penicillinase	75	61	130
$\gamma$ -Penicillinase	81	72	154

Relative enzymic activities are means of duplicate experiments done at a substrate concentration of  $10^{-3}$  M.

*Enzymic action.* Quantitative manometric estimation showed that 0.92 acid equivalents were produced from catalytic breakdown of benzylpenicillin by the  $\gamma$ -penicillinase, so that it is reasonable to suppose that penicilloic acid is the product of the reaction, as with the exo-enzyme. The relative rates of hydrolysis by  $\gamma$ -penicillinase of benzylpenicillin, penicillin K, phenoxymethylpenicillin and cephalosporin N did not differ significantly from those of the exo-enzyme (Table 3). Neither  $\gamma$ -penicillinase nor exo-penicillinase caused significant destruction of cephalosporin C (Dr E. P. Abraham; personal communication).

*Michaelis constant.* The initial velocities of penicillin destruction by purified exo-enzyme and by the  $\gamma$ -penicillinase preparation were both measured by the

bio-assay technique described in Methods, at initial penicillin concentrations of 12.5, 20 and 1000 units/ml. The results are plotted according to the method of Lineweaver & Burk (1934) and recorded in Fig. 3. It can be seen that there is no significant difference between  $\alpha$ - and  $\gamma$ -penicillinase whose Michaelis constants are calculated to be 28.5 and 27.8 units/ml., respectively. By this method the constant for the exo-enzyme is nearly 25 % lower than that obtained previously by a different technique (Manson, Pollock & Tridgell, 1954).

*The enzyme activity at different pH values.* The effect of hydrogen-ion concentration on the activity of the  $\gamma$ -penicillinase preparation is illustrated in Fig. 4, where values are plotted as a percentage of the maximum velocity obtained by using the iodometric assay and the same buffers as employed

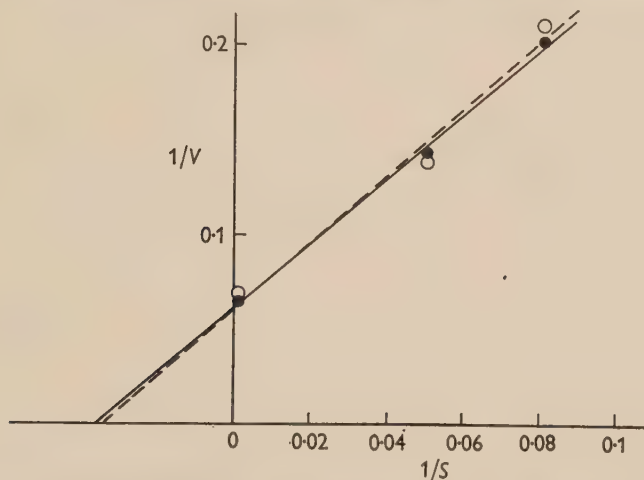


Fig. 3. Lineweaver & Burk plot for determination of Michaelis constants of exo-penicillinase (—●—●—) and  $\gamma$ -penicillinase (—○—○—) of penicillin-induced *Bacillus cereus* 569.  $S$ , initial penicillin concentration (units/ml.).  $V$ , rate of destruction of penicillin (units/ml./hr.).

previously (Manson *et al.* 1954) for a similar curve with the exo-penicillinase (also recorded for comparison in Fig. 4). Although the ( $H^+$ ) for optimum activity (pH 6.0) is the same for both enzymes, the activity of the  $\gamma$ -penicillinase decreased far more steeply on either side of the optimum value. The difference is clearly significant and seems unlikely to be due to impurities in the  $\gamma$ -penicillinase preparation, because no differences were detected between the pH/activity curves of purified exo-enzyme and a crude preparation of culture supernatant fluid. It should be noted that all the manometric assays of penicillinase have so far been done at pH 7.0, which is nearly the optimum for activity of the exo-enzyme, but would give values about 40 % below the optimum for  $\gamma$ -penicillinase. Thus, at pH 6.0, the  $\gamma$ -enzyme activities would be 66 % higher than those recorded here, while the activity of the exo-enzyme would remain unchanged.

*Sensitivity to iodine.* Penicilloic acid reacts specifically, and apparently stoichiometrically, with iodine to form a compound which does not produce a



blue colour with starch; this fact has been used as the basis for iodometric assay of penicillinase (Perret, 1954). A rapid approximate assay can be made by measuring the time required to decolorize a standard amount of iodine; and this can be done in the presence of iodine during the course of the reaction since the exo-enzyme is inactivated by the iodine relatively slowly under the conditions employed.  $\gamma$ -Penicillinase, when studied by this 'quick' iodine method, appeared to be enzymically completely inactive until it was discovered that the enzyme was destroyed almost instantaneously by the iodine. When the iodine was added later on, as an external indicator of the production of penicilloic acid, the enzymic hydrolysis of penicillin to penicilloic acid was

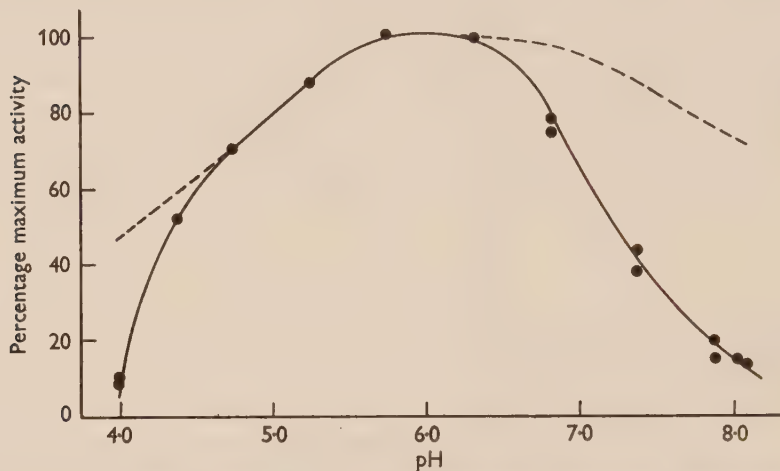


Fig. 4. Enzyme activity at different pH values for induced  $\gamma$ -penicillinase of *Bacillus cereus* 569, using a concentration of 6.7 units enzyme/ml. Results are plotted as percentage maximal activity (at pH 5.75). The dotted line shows the comparable curve obtained by Manson *et al.* (1954) for the induced exo-penicillinase from the same strain.

found to occur at the rate expected from manometric assay. This is a confirmation that penicilloic acid is the product of the reaction and shows that  $\alpha$ - and  $\gamma$ -penicillinases differ markedly in their reactions with iodine.

Dr N. Citri (unpublished experiments) has found that, in suspensions of organisms from cultures grown under normal conditions, the fraction of penicillinase activity which is not neutralized by antiserum to the exo-enzyme corresponds approximately to that which is rapidly inactivated by iodine. It therefore seems reasonable to conclude that, under the conditions employed, all unneutralizable activity—whether extractable or firmly bound to cell debris—is due to  $\gamma$ -penicillinase, and not to enzyme of the  $\alpha$ - $\beta$ -immunological type buried behind some structural barrier which prevents access of antibody.

*Immunological properties.* The fact that  $\gamma$ -penicillinase activity was not neutralized by anti-569 exo-penicillinase antibody did not necessarily mean that it could not specifically combine with that antibody. Combination might occur, as happens in certain specific enzyme-antibody reactions, without

neutralization. However, addition of  $10^4$  units  $\gamma$ -penicillinase to 0.5 ml. antibody solution produced no precipitate, while an enzymically equivalent quantity of exo-enzyme gave an obvious precipitate with the same amount of antibody. Moreover, addition to purified exo-enzyme of an enzymically equivalent quantity of the  $\gamma$ -penicillinase preparation did not modify the neutralization slope of the exo-enzyme by antibody. In other words, even in the presence of excess enzyme, the  $\gamma$ -penicillinase did not interfere with the reaction between exo-enzyme and antibody. It is therefore reasonable to conclude that  $\gamma$ -penicillinase does not combine with antibody prepared against exo-penicillinase.

Table 4. *Attempt to separate  $\gamma$ -penicillinase and exo-penicillinase of Bacillus cereus 569 by differential ultra-centrifugation for 2 hr. at 104,000 g*

Expt.	Layer	Vol. (ml.)	Total activity (units/ml.)	Percentage of total activity unneutralizable by antiserum
1 'Natural' mixture	Before centrifugation	13.5	665	30
	After centrifugation:			
	Top	3.0	415	23
	Bottom	1.0	975	29
2 Artificial mixture	Deposit	0.5	1120	37
	Before centrifugation	13.5	1640	20
	After centrifugation:			
	Top	1.9	1600	20.5
	Middle	9.0	1830	19
	Bottom	0.9	2800	20

*Differential ultracentrifugal analysis.* It was thought possible that a partial separation of soluble  $\beta$ - and  $\gamma$ -fractions obtained from preparations of disintegrated organisms might be achieved by the centrifugation of a mixture. Two such experiments were done: (a) on a 'natural' mixture obtained during the course of separation of the  $\gamma$ -penicillinase from an autolysate of organisms; (b) on an artificial mixture of the fully separated  $\gamma$ -penicillinase and pure exo-enzyme obtained from another large-scale preparation. Samples (13.5 ml.) were spun in the preparative head of the Spinco ultracentrifuge for 2 hr., at 104,000 g. Immediately after the run, samples were carefully pipetted from the top, middle and bottom layers with the minimum of disturbance, and assayed for total and unneutralizable activity; the results are shown in Table 4. It can be seen that there was a considerable sedimentation of enzyme, as shown by the higher activities in the lower layers. However, although there is some suggestion that the  $\gamma$ -enzyme in the 'natural' mixture had sedimented rather more rapidly than the neutralizable fraction, there was no significant separation of the two types in the artificial mixture. Thus it seems unlikely that their molecular weights are widely different.

*Attempts to transform  $\gamma$ -penicillinase*

It seemed possible that  $\gamma$ -penicillinase might be either a natural intracellular precursor of the  $\alpha$ - and  $\beta$ -fractions, or simply a complex formed by the  $\beta$ -fraction and some other substance present inside the organisms, this complex having biological and physico-chemical properties different from the  $\beta$ -enzyme. Attempts were therefore made to transform one type into the other both by *in vitro* and *in vivo* experiments. It was decided that loss in activity of the unneutralizable fraction associated with significant gain in activity of the neutralizable moiety or vice versa should be taken as *prima facie* evidence for interconversion. On this criterion, all results were negative. The following tests were done. *In vitro*: mixtures of approximately equal parts of neutralizable and unneutralizable penicillinase extracted from induced organisms were incubated alone, with trypsin and with deoxyribonuclease and ribonuclease for 1 hr. at 35°. No significant changes in the proportion of the two types occurred. *In vivo*: induced organisms were resuspended in fresh medium and the neutralizable and unneutralizable activity followed under conditions where total enzyme production was suppressed or markedly inhibited. These were: incubation at 35° (a) anaerobically in broth (argon bubbling through culture), (b) aerobically in broth with 20  $\mu$ g. chloramphenicol/ml., (c) aerobically in broth with  $8.3 \times 10^{-4}$  M-oxine, (d) aerobically in 0.1 M-glucose + 0.02 M-phosphate (pH 7.0) without added source of N. No decrease in activity of either the unneutralizable or neutralizable fractions took place.

*Inducibility of  $\gamma$ -penicillinase*

In un-induced cultures of *Bacillus cereus*, strain 569, about 50% of cell-bound penicillinase was found to be unneutralizable by excess antibody (as in induced cultures); and a proportion (about 30%) of the cell-bound unneutralizable basal enzyme could be extracted in a soluble form after the breaking of organisms in the Hughes press. It can therefore be concluded that  $\gamma$ -penicillinase, like exo-penicillinase, is present in a culture before induction with penicillin. After induction with 1 unit penicillin/ml., the unneutralizable cell-bound enzyme activity increased to constitute an approximately constant proportion (limits: 4.7–6.0%) of the total enzyme (see Fig. 5). Similarly, after induction with a suboptimal concentration of penicillin (0.1 unit/ml.), unneutralizable enzyme was found to constitute the same constant proportion (limits: 4.7–6.9%) of total penicillinase at all stages. It is quite clear, therefore, that  $\gamma$ -penicillinase formation is inducible by penicillin. Table 5 shows the quantities of total, of unneutralizable and of solubilized unneutralizable cell-bound penicillinase fractions found in cultures of un-induced organisms and in cultures harvested 40 min. after induction with 1 unit penicillin/ml. Even after this relatively short period of induction, there was an approximately 20-fold increase in all fractions, including the unneutralizable fraction which could be extracted in solubilized form. This increase is of the same order proportionally as the increase of exo-penicillinase under the same conditions.



Table 5 also shows that 83% of the cell-bound penicillinase activity of the constitutive-penicillinase *Bacillus cereus* mutant 569/H was not neutralizable by excess antiserum and that 45% of this unneutralizable fraction was extracted in a soluble form after disintegration of the organisms, as with strain 569. The 569 (induced) and the 569/H (constitutive) exo-penicillinase have

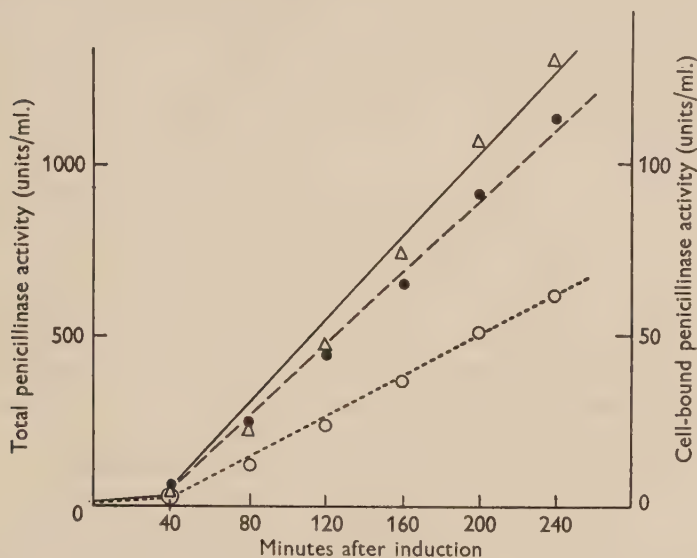


Fig. 5. Formation of the different fractions of penicillinase in a culture of *Bacillus cereus* 569 growing in 1% (w/v) gelatin broth following induction with 1 unit penicillin/ml. In order to follow enzyme production over a long period a sample of the culture was diluted with an equal volume of fresh medium every 40 min.; and results are expressed as total enzyme production in terms of the original culture (viz. activity/ml.  $\times$  dilution factor). — $\triangle$ — $\triangle$ —, total penicillinase; -- $\bullet$ -- $\bullet$ --, cell-bound penicillinase;  $\cdots$   $\circ$   $\cdots$   $\circ$   $\cdots$ , fraction of cell-bound penicillinase not neutralizable by antiserum to exo-enzyme.

Table 5.  $\gamma$ -Penicillinase:inducibility in *Bacillus cereus* 569 and its presence in *Bacillus cereus* 569/H (constitutive-penicillinase mutant strain)

Strain	Cell-bound penicillinase		
	Total	Unneutralizable by antiserum to exo-enzyme	Fraction of un-neutralizable activity extractable into solution after cell disintegration
			(= $\gamma$ -penicillinase)
			Penicillinase activity (units/mg. dry wt. organisms) .
<i>Bacillus cereus</i> 569:			
Not induced	0.64	0.23	0.11
40 min. after induction with 1 unit penicillin/ml. at concentration of organisms corresponding to 0.3 mg. dry wt./ml.	13.10	6.10	2.60
<i>Bacillus cereus</i> 569/H (constitutive-penicillinase mutant strain)	167	137	61.7

been shown (Kogut *et al.* 1956) to be physico-chemically and immunologically indistinguishable. Thus, although no further studies have been made on the soluble unneutralizable penicillinase of strain 569/H, it seems probable that it is identical with the  $\gamma$ -penicillinase of strain 569; and that, in this respect as in all others investigated, strain 569/H behaves, in the absence of added penicillin, in the same manner as strain 569 after induction with penicillin. In contrast to strain 569/H, little, if any  $\gamma$ -penicillinase was found in the other constitutive-penicillinase strain of *Bacillus cereus*, 5/B (Pollock, Torriani & Tridgell, 1956; Sneath, 1955). Less than 7% of the total cell-bound activity of strain 5/B failed to react with antiserum.

*Test for the presence of  $\gamma$ -penicillinase in cell-free supernatant fluid from a culture of Bacillus cereus*

$\gamma$ -Penicillinase is not normally liberated to any significant extent into the medium. This was shown by an experiment in which 1.0 ml. of untreated supernatant fluid (containing 1550 units of enzyme) from a culture of the 'constitutive-penicillinase' *Bacillus cereus* mutant, strain 569/H, was mixed with a gross excess of antibody (0.25 ml. anti-569  $\alpha$ -penicillinase serum containing a total of 5500 neutralization units of antibody; see Pollock, 1956). The mixture was incubated overnight at 35°, and the supernatant fluid, after removal of the precipitate by centrifugation, was found to contain only 5.5 units, or 0.35% of the original penicillinase activity. The unneutralizable activity contained in the cells from 1.0 ml. of the same culture was found to be 86 units. It can safely be assumed that no significant quantity of  $\gamma$ -penicillinase was carried down with the precipitate formed between the  $\alpha$ -enzyme and the anti- $\alpha$ -penicillinase antibody. This is clear from an experiment in which 1.0 ml. of the same culture supernatant referred to above (containing 1550 units of penicillinase) was mixed with 275 units of purified  $\gamma$ -enzyme and 0.1 ml. of antiserum (=2200 neutralization units: 50% more than that required to combine with all the  $\alpha$ -enzyme present in the culture supernatant). After centrifuging down the precipitate which formed during 16 hr. of incubation at 35°, no loss of  $\gamma$ -penicillinase activity from the supernatant fluid was observed.

The 5.5 units of activity left after precipitation of the exo-enzyme with excess antibody might have been due either to the residual activity of a small amount of  $\alpha$ -enzyme/antibody complex still remaining in solution (the more likely explanation) or to traces of  $\gamma$ -penicillinase itself. However, even assuming that it were all  $\gamma$ -penicillinase, it would only represent 6% of that found fixed to the organisms, thus indicating the maximum possible proportion liberated into the medium from a growing culture.

#### DISCUSSION

There are several other instances, analogous to that of the  $\alpha$ - and  $\gamma$ -penicillinases of *Bacillus cereus*, where a protein formed by some tissue or population of cells occurs in two or more closely related, but physico-chemically or immunologically distinct, forms having apparently similar biological actions (pan-

creatic ribonucleases, Martin & Porter, 1951; heart lactic dehydrogenases, Neilands, 1952; beef insulins, Harfenist, 1953; beef chymotrypsins, Jacobsen, 1947; ox liver catalases, Brown, 1952). In none, however, is there any information on possible differences in localization of the fractions in relation to cell structure. Also, in general, the observed differences have been finer than those found between the two types of penicillinases described here. One of these penicillinases—the  $\gamma$ -enzyme—appears to be exclusively cell-bound. Indeed, since  $\gamma$ -penicillinase can only be obtained from organisms in a soluble form after breaking them in a Hughes press, it seems reasonable to consider it 'intracellular'. This is, however, primarily an operational denotation. Its rapid appearance in the supernatant fluid, obtained by centrifuging the debris after disruption of the organisms, suggests that at least a proportion of the  $\gamma$ -enzyme exists in the cytoplasm, and is thus immediately liberated by damage to the cell wall. But the possibility that the enzyme is normally bound on the outside of the organisms and is freed into solution by mechanical disintegration of their supporting structure, though rather unlikely, has not been formally excluded.

$\gamma$ -Penicillinase is distinguishable from  $\alpha$ - and  $\beta$ -penicillinase by its immunological reactions, the shape of the pH/activity curve, its greater solubility in ammonium sulphate and its relatively rapid inactivation by iodine. This property of iodine-sensitivity has been used to show that the fraction of cell-bound penicillinase which is not neutralized by antibody to exo-enzyme is probably all  $\gamma$ -penicillinase.

Since it is possible to wash off from whole organisms most of the enzyme that is neutralized by antibody ( $\beta$ -fraction), it seems likely that in normal growing cultures the  $\alpha$ -fraction is derived directly from the  $\beta$ -fraction. In other words, it is probable that penicillinase of the  $\alpha$ - $\beta$  immunological type first occurs in an active form as cell-bound enzyme, and that immediately or very shortly after formation it appears on the surface of the organisms in a position sufficiently exposed to allow combination with antibody. Shortly after that, one may suppose that it is liberated into the medium as normal exo-enzyme. In any case, it is reasonable to conclude that  $\beta$ -penicillinase is the normal precursor of  $\alpha$ -penicillinase.

The relationship of  $\gamma$ -penicillinase to the other two fractions is less clear. Three of the most likely alternatives are illustrated in Fig. 6. All reactions have been assumed to be irreversible. In scheme 1,  $\gamma$ -penicillinase and the exo-enzyme are visualized as being formed by independent mechanisms. But this takes no account of the similarity in the substrate-combining groups (as indicated by identical Michaelis constants) or the constant proportion of unneutralizable to total penicillinase activity formed in un-induced cultures at all stages after induction with two different concentrations of penicillin. Both of these facts suggest that the specific mechanisms for the formation of the two types of enzyme may be closely related at one point in the sequence of necessary reactions.

Scheme 2 is not supported by the failure, hitherto, of all attempts to demonstrate conversion of unneutralizable penicillinase to neutralizable penicillinase.



Scheme 3, in which no interconversion of  $\gamma$ - and  $\beta$ -penicillinase would be expected, accounts for the similarities in properties and rates of production of the two types of enzymes by postulating the existence of a common specific penicillinase precursor (not essential to the other two schemes) for which there is already some indirect evidence (Pollock, 1953). However, although at the moment the facts appear to favour scheme 3, further information on the relative rates of synthesis of the different fractions under a wide variety of conditions is needed before deciding which hypothesis is the most probable.

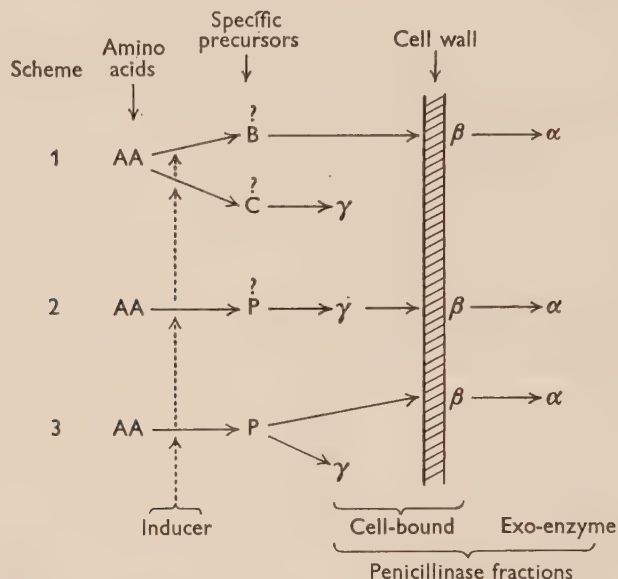


Fig. 6. Three alternative schemes to illustrate the possible metabolic relationships between the  $\alpha$ -,  $\beta$ - and  $\gamma$ -penicillinases of *Bacillus cereus* 569.

I wish to thank Mr J. Orr for carrying out the ultracentrifuge runs. I am grateful to Dr E. P. Abraham for a gift of cephalosporin N. I am also deeply indebted to Miss Joan Fleming for expert technical assistance.

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## Control of Sexual Activity in *Chlamydomonas* by Light

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**SUMMARY:** *Chlamydomonas moewusii* is a unicellular, haploid, heterothallic alga. Suspensions containing a high proportion of active gametes are readily obtainable. The species is obligately photo-autotrophic. Organisms grown in light lose sexual activity when incubated in darkness, and only regain it when illuminated. Organisms of both mating-types require light for sexual activation. This reaction appears independent of the presence or absence of CO<sub>2</sub>. It was not found possible to replace the effect of light by the addition of any chemical or natural extract to the medium. By the use of a simple device, changes in the sexual activity of a given suspension were followed experimentally. The sensitivity of organisms to light remained approximately constant during an experimental run of 1-2 hr. Mating activity of organisms transferred to darkness remained constant for a few minutes ('dark lag') before decreasing. The length of the dark lag was a function of the duration of previous illumination. The dark lag of *plus* organisms was shorter than that of *minus* organisms under comparable conditions. After the lag period, decline of activity in darkness was approximately exponential. Rates of loss of activity between 10° and 35° were measured, and half-life values calculated, for each mating-type. The rate of loss of activity was approximately the same for both mating-types. Loss of sexual activity was somewhat accelerated by anaerobiosis. Resumption of sexual activity on re-illumination followed a short period of persistent inactivity ('light lag'). The length of the light lag was not dependent on the duration of the preceding dark period. At 25°, the lower threshold of white light was 10 f.c. for *minus* organisms, 50 f.c. for *plus* organisms. At 300 f.c., *minus* organisms were reactivated between 12.5° and 35°, *plus* organisms between 17.5° and 30°. The action spectrum of photoactivation exhibited two peaks, around 450 and 680 mμ., thereby resembling the absorption spectrum of the chloroplast. Phenylurethan ( $6 \times 10^{-4}$  M) reversibly inhibited photosynthesis and photoactivation of sexuality, while respiration and the mating process itself were affected to a much lesser degree. It is postulated that mating activity is controlled by an intracellular hormone, activated at the plastid during illumination, and operative at the flagella.

Light has long been known to play an important role in the sexual activity of plants. In the case of *Chlamydomonas* and related algae, experimental observations on the action of light have been made by Klebs, Moewus, Smith and others: their work has been briefly reviewed elsewhere (Lewin, 1954). The following experiments were carried out solely with the species *Chlamydomonas moewusii* Gerloff, which lends itself particularly well to studies of sexual activity for the following reasons: (1) Methods have already been evolved (Lewin, 1953*b*) for regularly eliciting the abundant production of sexually active organisms. (2) The species is heterothallic, so that mating can be initiated at a given time by mixing suspensions of organisms of complementary mating-types. (3) Since paired organisms remain *in copulo*, without further fusion, for some hours, the number of pairs provides a convenient



measure of the activity of gamete suspensions. (4) The loss of sexual activity in darkness, and reactivation of gametes by light, are processes which take place within a matter of minutes, so that many experimental investigations can be carried out without the added complications of cell ageing or cell division.

The following experiments were designed to obtain more precise quantitative information on the dynamics of these processes, and thereby to find some indications as to the chemical reactions underlying sexual activity.

#### METHODS

The strains of *Chlamydomonas moewusii* used in these investigations were originally isolated by L. Provasoli in 1948. Cultures of opposite mating-type, designated as *plus* and *minus* respectively, were maintained separately on agar slopes, growth in each being exclusively vegetative. Since sexual activity appears to be confined to young flagellated organisms recently liberated after vegetative reproduction, the following empirical method was evolved for the production of active gamete suspensions. The basal mineral medium employed (MM) contained (g./l.):  $K_2HPO_4$ , 0.2;  $MgSO_4 \cdot 7H_2O$ , 0.2;  $Ca(NO_3)_2 \cdot 4H_2O$ , 1.0, supplemented with Fe and other trace elements (Lewin, 1953*b*). For growth of gametes (MAC), MM medium was supplemented with (g./l.): Na acetate  $2H_2O$ , 1.0;  $Na_3$  citrate  $2H_2O$ , 1.0; agar (Difco), 10.0. (1) Erlenmeyer flasks (125 ml.) containing 30 ml. medium MAC, and covered by an inverted 60 ml. beaker, were sterilized by autoclaving. After cooling, the agar surface was inoculated by pouring on a dense culture of organisms grown in mineral medium MM, and then decanting the free liquid. Two flasks were prepared in this way, one for each mating-type. The cultures were illuminated at *c.* 350 f.c. under 'white' fluorescent tubes for 7 days. (2) They were then transferred to darkness and incubated for 18 hr., during which time division of each enlarged vegetative organism gave rise to 4–8 young organisms with gametic activity. (3) Such surface cultures were then flooded with 15 ml. of a weak buffer solution FM (g./l.:  $Na_2HPO_4 \cdot 7H_2O$ , 0.2;  $CaCl_2 \cdot 2H_2O$ , 0.05; pH 8.0), and incubated for 1 hr. in darkness. Under these conditions abundant flagellated and motile organisms were released into the medium (Lewin, 1953*a*), producing a suspension containing 5 to  $10 \times 10^6$  organisms/ml. (4) Suspensions prepared in this way were sexually inactive. For experiments on the loss of activity in darkness (p. 174), it was first necessary to activate the organisms by *pre-illumination*. In such cases the suspension to be tested was decanted into a clean flask and illuminated under the fluorescent lights for 30–120 min.

In order to follow the behaviour of *plus* and *minus* organisms separately, the following procedure was adopted. Briefly, it consisted of subjecting organisms of one mating-type to various conditions of light and temperature in a simple reaction chamber (Fig. 1 and Pl. 1), and at intervals removing samples in which mating activity was tested against a standard suspension of gametes of the opposite type.

Uniform mating suspensions of *plus* and *minus* organisms were used for each experiment. Samples (0.3 ml.) of organisms of one mating-type were pipetted

into a series of small tubes  $75 \times 8$  mm. internal diameter in a Perspex holder, and were maintained in a fully light-activated state by constant illumination under fluorescent tubes. Such suspensions will be referred to as 'tester' stocks. Organisms of the complementary mating-type to be tested were drawn by suction into the reaction chamber *F* (see Fig. 1) through opening *D*, and were kept stirred by a slow stream of air or other gas, tap *C* being open. At intervals the tap was closed temporarily, and a small sample (2 drops, *c.* 0.1 ml.) expelled from *D*, by the use of a syringe connected at *A*, into one of the tubes of tester stock *G*. Pairing was generally completed in 10–15 min. (see Fig. 2) and the pairs remained motile without fusing for an indefinite period when light was excluded. The mixed suspensions were therefore left for an hour or more in

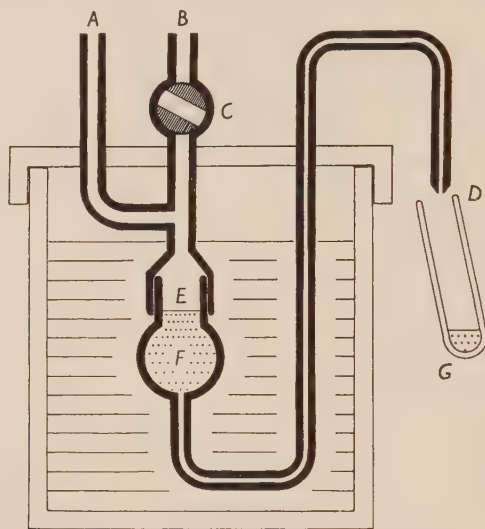


Fig. 1. Diagram of reaction chamber. *A*, to syringe; *B*, to aspirator; *C*, tap; *D*, air intake or delivery outlet for suspension; *E*, standard-taper ground joint; *F*, suspension of organisms subjected to various temperature and illumination treatments; *G*, tube with tester organisms of opposite mating-type.

darkness, and the organisms were then killed by the addition of a drop of 1% aqueous iodine. Mating activity was determined by counting the number of copulating pairs in a given volume of liquid in a haemocytometer.

For the determination of an action spectrum (p. 179), Bausch & Lomb interference filters were used to screen light from an incandescent tungsten filament in a small projection lantern. The transmission of such filters, as measured in a Beckman spectrophotometer, is restricted to a narrow band, the intensity falling to about 10% at 20  $m\mu$ . on either side of the peak. The distance between the organism suspension and the light source was adjusted for each filter so that the incident light energy, as measured by a thermocouple and mirror galvanometer, was of the same value (*c.* 500 ergs/cm.<sup>2</sup>/sec).

Cultures were grown and maintained at  $22^\circ \pm 1^\circ$ , except in the reaction chamber, which was immersed in a water-bath thermostatically controlled at various temperatures with a fluctuation range of less than  $\pm 0.1^\circ$ .

## RESULTS

*Organism pairs as a measure of gamete activity*

It may be postulated that, in the presence of an excess of active *minus* gametes, every *plus* gamete can ultimately pair if its sexual activity is retained long enough for it to find a partner. Under such conditions, the number of pairs formed will thus be a measure of the activity of gametes in the original *plus* suspension. *Mutatis mutandis*, the same would be expected for *minus* gametes, which are indistinguishable in size and activity. An indication of the proportionality between the numbers of active gametes and of pairs of organisms

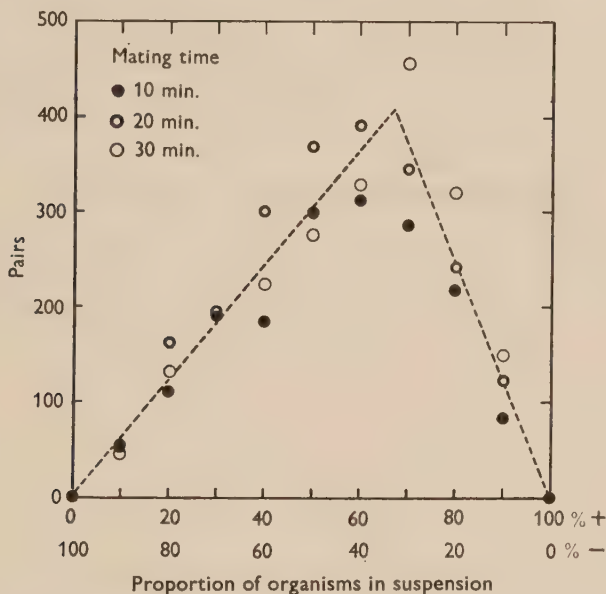


Fig. 2. Effect of relative proportions of gametes on numbers of pairs/ $\mu$ l. *Plus* suspension, 1600 organisms/ $\mu$ l. *Minus* suspension, 1800 organisms/ $\mu$ l. Organisms killed 10, 20, 30 min. after mixing.

was obtained from the following experiment. Samples from illuminated *plus* and *minus* suspensions containing approximately equal numbers of organisms were mixed in various proportions in light. After 10, 20 and 30 min., samples were removed, and the organisms killed with iodine. Counts of pairs of organisms indicated that when active *plus* gametes were in a minority, the number of pairs was proportional to the number of *plus* gametes: while when the latter were in excess, the number of pairs was proportional to the *minus* gametes. This is shown in Fig. 2, in which two straight lines indicating direct proportionality intersect at a *plus:minus* ratio of organisms where there are presumably equal numbers of active gametes in the mixture. The fact that in this experiment the maximum number of pairs occurred in a mixture containing 70% *plus* and 30% *minus* organisms indicated that, in the suspension of



*plus* organisms used, there happened to be a lower density of active gametes than in the *minus* suspension. It also appeared from this figure that mating was virtually completed within 10 min. of mixing the suspensions.

#### *Loss of sexual activity in darkness*

The effects of illumination and darkness on a gamete suspension in the reaction chamber are shown in Fig. 3, which represents the results of a typical experiment. Similar curves were obtained when either *plus* or *minus* organisms were studied, indicating that for mating to take place both mating-types require illumination, and that both types lose activity in darkness. Since, with certain reservations, mating activity appeared to be lost at an exponential rate, pair numbers were expressed in a semi-logarithmic plot against time in darkness to give approximately a straight line. The slope of this line provided a measure of the rate of decay of mating activity under the conditions investigated. Fig. 4 illustrates the results of two experiments plotted in this way.

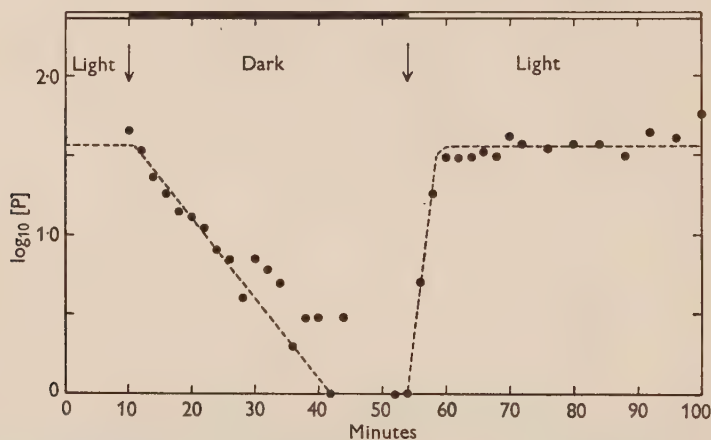


Fig. 3. Effect of darkness and light on sexual activity. *Minus* organisms tested. Pre-illumination 30 min., 300 f.c. Temperature 23°.  $P$  = pairs/0.3  $\mu$ l.

In organisms which had been transferred to darkness after pre-illumination, the decay rate of mating activity was found to be dependent on the temperature at which they were maintained in the dark. The results of an extended series of experiments carried out at various temperatures between 10° and 35° are summarized in Table 1, in which the slopes have been expressed as half-life values. It was not practicable to investigate temperatures below 10°, since such experiments would last for several hours, during which time multiplication and other changes in the illuminated suspensions (see p. 178) would tend to change the sexual activity within the suspensions to an appreciable extent.

The rate of decay was unaffected by turbulence, but was found to be somewhat accelerated in the absence of oxygen (Fig. 5). The passage of a stream of CO<sub>2</sub>-free air did not appreciably affect the course of the reaction (Fig. 6).

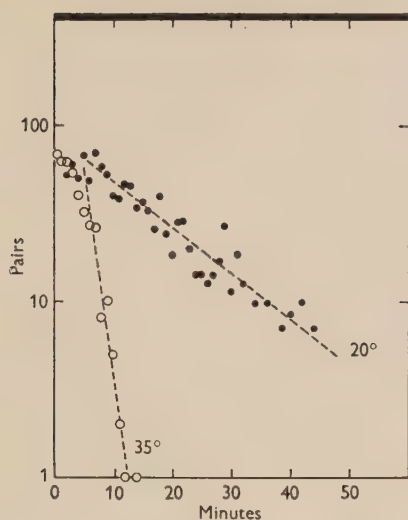


Fig. 4.

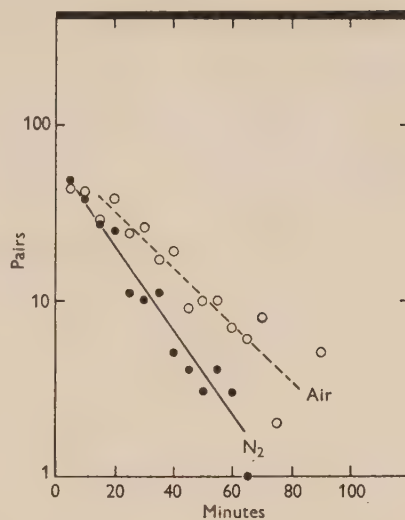


Fig. 5.

Fig. 4. Effect of temperature on loss of sexual activity in darkness. *Plus* organisms tested. Pre-illumination 60 min., 300 f.c. Pairs/0.5  $\mu$ l.

Fig. 5. Effect of oxygen on loss of sexual activity in darkness. *Plus* organisms tested. Pre-illumination 30 min., 300 f.c. Temperature 23°. Pairs/0.3  $\mu$ l.

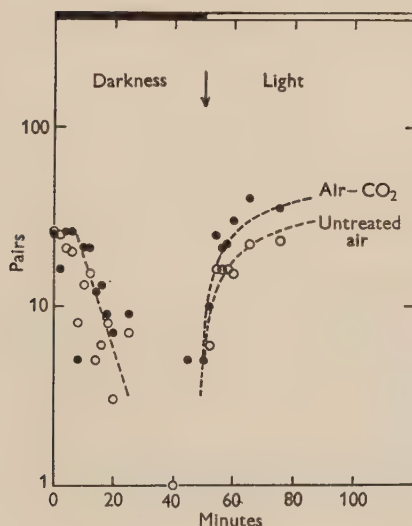


Fig. 6.

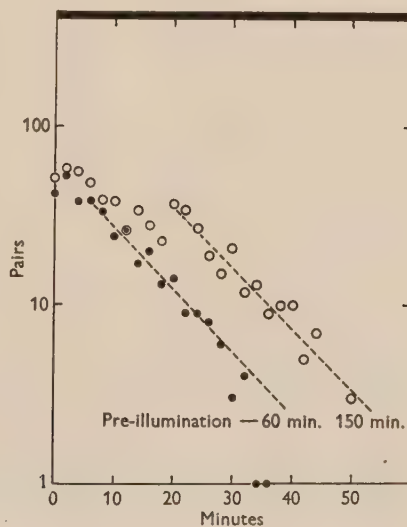


Fig. 7.

Fig. 6. Effect of presence of  $\text{CO}_2$  on activation of sexuality by light. *Minus* organisms tested. Pre-illumination 30 min., 300 f.c. Temperature 24°. Pairs/0.3  $\mu$ l.

Fig. 7. Effect of pre-illumination period on loss of sexual activity in darkness. *Minus* organisms tested. Pre-illumination 300 f.c. Temperature 23°. Pairs/0.3  $\mu$ l.

Organisms which received only a short period of pre-illumination began to lose sexual activity as soon as they were transferred to darkness; whereas organisms illuminated for a longer period retained full mating activity in darkness for several minutes before the numbers of pairs began to fall off (Fig. 7). The lengths of the lag period observed after various durations of pre-illumination at 220 f.c. are given in Table 2. The significance of this lag period is discussed below (p. 183).

Table 1. *Loss of mating activity in darkness: half-life values*

Strain	Pre-illumination (min.)	Temperature (° C.)					
		10	15	20	25	30	35
		Half-life values (min.)					
<i>Plus</i>	30	33	14	8	5	3	1.33
	60	32	19	6.5	6	3	1.33
	90	48	17.5	6.5	5.5	3.75	1.33
	120	36	18	7	6	4	1.5
<i>Minus</i>	30	52	33	10	8	2.75	1.5
	60	45	32	15	9	3.75	1.5
	90	49	33	12	19	3	1.75
	120	35	28	11	7	4.5	1.75

Table 2. *Loss of mating activity in darkness: duration of lag*

Strain	Pre-illumination (min.)	Temperature (° C.)					
		10	15	20	25	30	35
		Duration of lag (min.)					
<i>Plus</i>	30	45	10	5	4	4	2
	60	65	19	9	6	11	5
	90	70	25	10	7	8	3
	120	72	30	19	10	10	4
<i>Minus</i>	30	180	50	19	10	3	2
	60	180	145	20	25	15	3
	90	204	125	37	42	22	6
	120	175	100	48	43	29	5

#### *Restoration of sexual activity by light*

At intensities of white light around 200–300 f.c., reactivation of gamete suspensions was rapid, usually being almost completed in 15–20 min.; at lower intensities, pairing activity increased more slowly (Fig. 8). At 25° the lower threshold for light reactivation was of the order of 10 f.c. for *minus* organisms, and 50 f.c. for *plus* organisms. With the illumination constant at 300 f.c., *minus* organisms were reactivated in the temperature range 12.5°–35° (Fig. 9): in the case of *plus* organisms the range was more restricted, 17.5°–30° (Fig. 10).

The presence of CO<sub>2</sub> appeared to be unnecessary for the process of sexual activation by light. Parallel samples from a uniform gamete suspension were incubated for 1 hr. in darkness, one being aerated with normal air, and the other with air from which the CO<sub>2</sub> had been scrubbed by passage through two



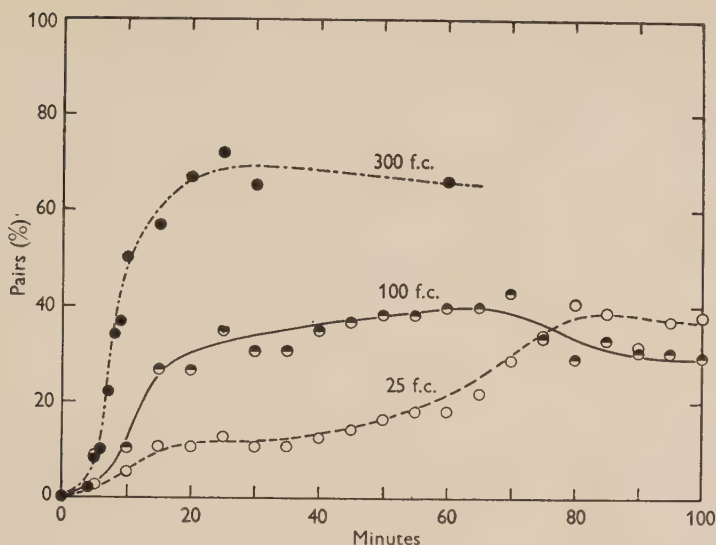


Fig. 8. Effect of light intensity on activation of sexuality. *Minus* organisms tested. Illumination 25, 100, 300 f.c. respectively. Temperature 25°. Pairs expressed as percentage of 'maximum' obtained in comparable gamete mixtures illuminated at 300 f.c. for 2 hr. Max. numbers, respectively, 182, 254, 177 pairs/0.5  $\mu$ l.

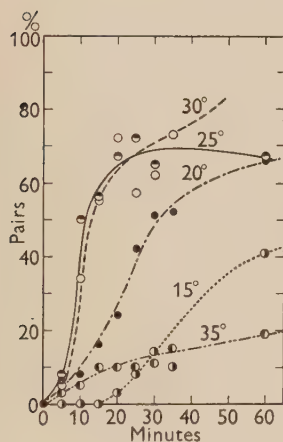


Fig. 9.

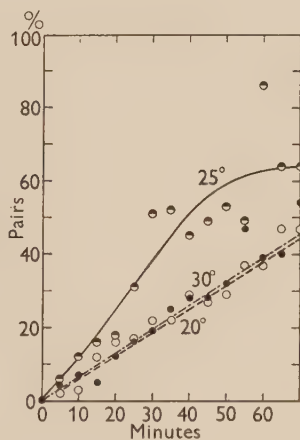


Fig. 10.

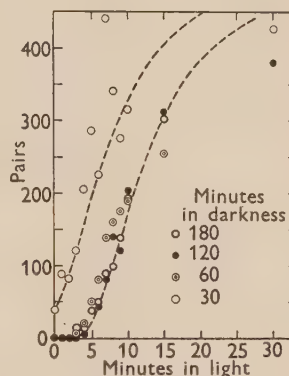


Fig. 11.

Fig. 9. Effect of temperature on activation of sexuality of *minus* organisms by light. Illumination 300 f.c. Temperatures 15°, 20°, 25°, 30°, 35°, respectively. Pairs expressed as percentage of 'maximum': see Fig. 8. Max. numbers, respectively, 79, 246, 177, 110, 182 pairs/0.5  $\mu$ l.

Fig. 10. Effect of temperature on activation of sexuality of *plus* organisms by light. Illumination 300 f.c. Temperatures 20°, 25°, 30° respectively. Pairs expressed as percentage of 'maximum': see Fig. 8. Max. numbers respectively 57, 51, 87 pairs/0.5  $\mu$ l.

Fig. 11. Effect of duration in darkness on subsequent reactivation of sexuality by light. Illumination 300 f.c. Temperature 24°. Pairs/0.2  $\mu$ l.

flasks containing 10% (w/v) sodium hydroxide. They were then illuminated without altering the aeration system, and samples were removed at intervals and tested for sexual activity. No significant difference was observed between the two activation curves, either in the rate of activation or in the level of maximum activity (Fig. 5).

Organisms in which sexual activity had been diminished, but not eliminated, by a short period in darkness showed an immediate increase in activity on illumination. Once sexual activity had been completely lost, the reactivation of gametes by light followed a short lag period, which was of similar duration in organisms subjected to as little as 30 min. of darkness, or as much as 180 min. (Fig. 11).

#### *Variation in sensitivity to light*

In spite of precautions to obtain as uniform a suspension of gametes as possible (see p. 171), there doubtless remained some variation in the sensitivity of individual organisms to stimulation by light. In addition, it was necessary to determine to what extent experimental results might be influenced by fluctuations of overall activity in suspensions maintained in darkness under turbulent conditions in the reaction chamber. Experiments of the following type were therefore carried out. Organisms were introduced into the chamber

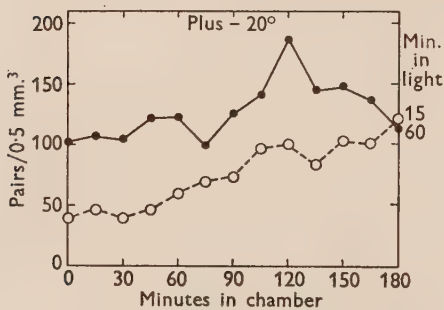


Fig. 12.

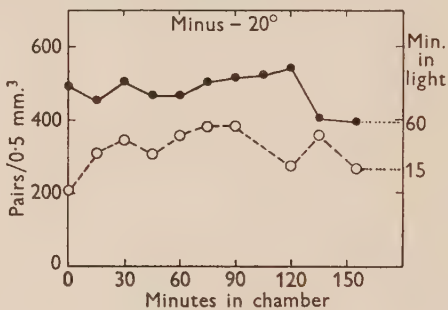


Fig. 13.

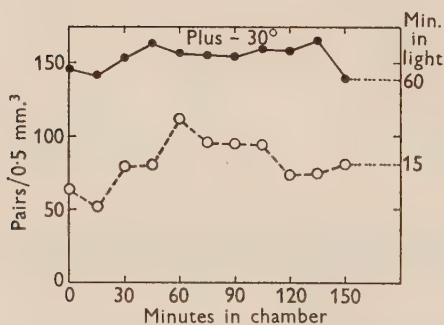


Fig. 14.

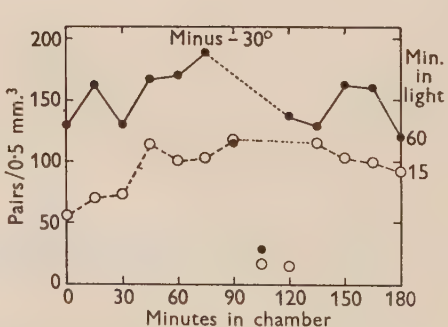


Fig. 15.

Figs. 12–15. Effect of experimental conditions on sexual response to a constant dose of light. Illumination 300 f.c.: duration 15 or 60 min. Fig. 12, *plus* organisms tested. Temperature 20°. Fig. 13, *minus* organisms tested. Temperature 20°. Fig. 14, *plus* organisms tested. Temperature 30°. Fig. 15, *minus* organisms tested. Temperature 30°.

and maintained in darkness. At 15 min. intervals, samples were removed, mixed with tester stock (fully light-activated organisms of the complementary mating-type), and then subjected to a standard dose of illumination (15 or 60 min. at 300 f.c.). The mixtures were then transferred to darkness for the completion of the mating reaction. The results of four such experiments, carried out on each mating type at 20° and 30° respectively, are shown in Figs. 12–15. They indicated that over a period of hours there was some fluctuation in the overall sensitivity of gamete suspensions to light.

The causes underlying these variations of response may be complex. Organisms of both mating-types were ageing, those of the tester stock in light, the others in a turbulent aerated suspension in darkness. Not only did the number of potential gametes change with time, but apparently also the threshold value of light required for sexual activation. This emerged from the observation that the proportion of gametes capable of sexual activation was higher in suspensions receiving 1 hr. illumination than in those illuminated for only 15 min.

Such changes of gametic sensitivity to light certainly occurred in all experiments. However, it may be noted that the drift in potential sexual activity was a gradual one, evident over a period of some hours, whereas the experiments described in the sections on 'variation in sensitivity to light' and 'action spectrum' occupied considerably shorter periods. Considering the magnitude of variation between replicate runs, the error due to drift of intrinsic sexuality can probably be ignored at this stage.

#### Action spectrum

A 5 min. period of dim illumination was insufficient for complete reactivation of gametes (Fig. 8), and under such circumstances sexual activity became a function of light intensity. Samples of a uniform mixed suspension containing approximately equal numbers of dark-inactivated *plus* and *minus*

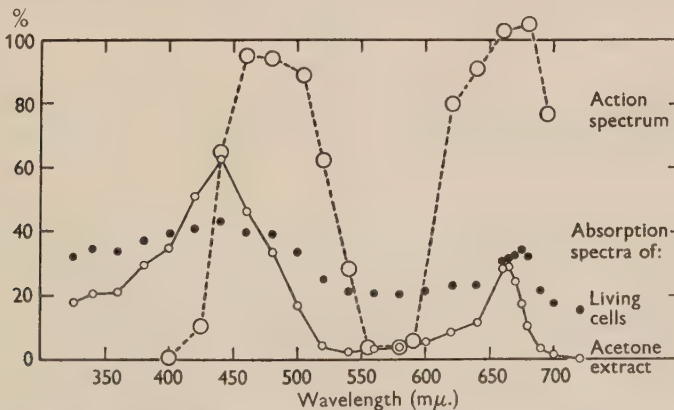


Fig. 16. Action spectrum of sexual activation by light. *Plus* and *minus* organisms mixed after 3 hr. in darkness. Temperature 20°. Pairs expressed as percentage of 'maximum' obtained in sample illuminated for 5 min. in white light, then incubated in darkness. Max. 146 pairs/0.2  $\mu$ l. Light absorptions determined in Beckman spectrophotometer: 'absorption' of living organisms not compensated for scattered light.



organisms were irradiated for 5 min. with light transmitted through interference filters, and then kept in darkness until after pairing ceased. By comparing the effectiveness of various wavelengths of light in inducing pairing, it was possible to obtain an action spectrum of sexual activation. The form of the curve, with peaks around 450 and 680  $m\mu$ ., somewhat resembles the absorption spectrum of the plastid pigments (Fig. 16), suggesting that chlorophyll is responsible for mediating the light reaction of mating, as well as that of photosynthesis. The curve should not be interpreted too closely, however, since (a) the quantum dosages employed were not equivalent for each filter, being inversely proportional to wavelength, and (b) the linearity of the relation of cell-pair numbers to incident light intensities within the range of the experiment was not established.

#### *Action of inhibitors on the light reaction*

Since the action spectrum implicated chlorophyll as the sensitizing pigment, some interrelation between photosynthesis and sexual activation by light seemed probable. For this reason the influence of photosynthesis inhibitors upon these two processes was investigated. Phenylurethan, which is thought to inhibit the primary photochemical reaction, proved most useful in this

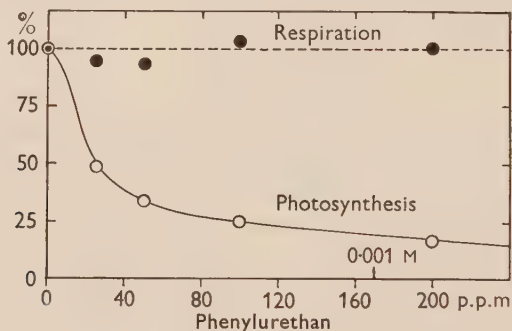


Fig. 17. Effect of phenylurethan on relative rates of respiration and photosynthesis. *Plus* organisms tested. Illumination 500 f.c. Temperature 25°. For further experimental details, see Lewin & Mintz, 1955.

respect (Lewin & Mintz, 1955). In certain concentrations of this narcotic (e.g.  $6 \times 10^{-4} M$ ) the organisms remained viable and motile, exhibiting normal or slightly augmented respiration, whereas photosynthesis was markedly inhibited (Fig. 17). It was found that this concentration of phenylurethan did not severely inhibit mating of gametes which had already been sexually activated by light; whereas gametes which had been incubated in darkness regained little sexual activity when illuminated in the presence of this narcotic (Fig. 18). No other agent among reported inhibitors of photosynthesis (see Lewin & Mintz, 1955) was found to exhibit a similar differential inhibition. When the phenylurethan was removed by washing, the organisms regained both their power of photosynthesis and their ability to become sexually

reactivated by light. The parallel effects of phenylurethan, on photosynthesis and on sexual reactivation, offer further support for the hypothesis that these processes have some photochemical reaction in common.

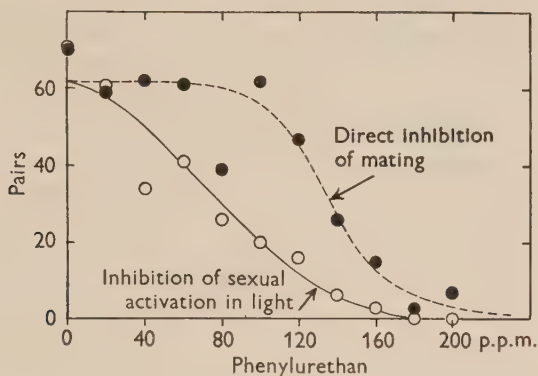


Fig. 18. Effect of phenylurethan on sexual activation in light. Darkened *plus* and *minus* suspensions mixed. Illumination 300 f.c., 60 min. Temperature 23°. Pairs/0.3  $\mu$ l. Direct effect of inhibitor on mating was determined by using activated organisms, which had received 60 min. pre-illumination, in a parallel series.

#### DISCUSSION

The environmental factors governing the formation of gametes in *Chlamydomonas moerwusii* closely parallel those which control the production of 'dark cells' in *Chlorella ellipsoidea* (Tamiya, Iwamura, Shibata, Hase & Nihei, 1953); and it seems likely that these are homologous stages in the two genera, although sexual reproduction has not been reported in *Chlorella*.

The experiments reported in this paper were carried out using the number of pairs as a measure of sexual activity. Recently, Förster & Wiese (1954*a, b*), using *Chlamydomonas eugametos*, demonstrated that cell-free filtrates from suspensions of active gametes of one mating-type may induce a clumping reaction among organisms of the complementary type. This kind of response indicates the presence of a sex-substance of type 2 (Lewin, 1954). It might be well to point out that, in the methods employed and in the activity demonstrated, the work of Förster & Wiese has followed different lines from those reported here.

It is convenient, in discussing the effects of light on *Chlamydomonas* gametes, to postulate that mating activity is controlled by a hormone, a chemical agent which is elaborated in one part of the organism, presumably at the chloroplast, and which affects activity at another site, the flagellum (Lewin, 1954). There is some evidence that this hormonal agent is not liberated into the medium, since all attempts to induce sexual activity in gametes incubated in darkness, by adding cell-free filtrates from illuminated organisms, were unsuccessful. Förster & Wiese (1954*b*) discussed the technical difficulties inherent in experiments attempting to demonstrate the effect of such a soluble agent (referred to by them as a B-type gamone, and by Lewin (1954) as sex-substance activity of type 3). Likewise it was not found possible to delay the loss of activity in darkness, or to induce mating activity of darkened organisms, by the passage of

oxygen, or by the addition of chemical agents, tested over a wide range of concentrations; these included adenosine triphosphate, phosphoglycerate, diverse sugars and sugar phosphates, glutathione, cysteine, and various organic acids such as acetate and succinate.

The ability of the organisms to pair seems to fall-off in darkness at an exponential rate, an approximately linear relationship being found in any one experiment between time and the logarithm of the number of gamete pairs. This would indicate that the loss of mating activity in each organism might be attributed to a single event, such as the decay of a particle, with a fixed

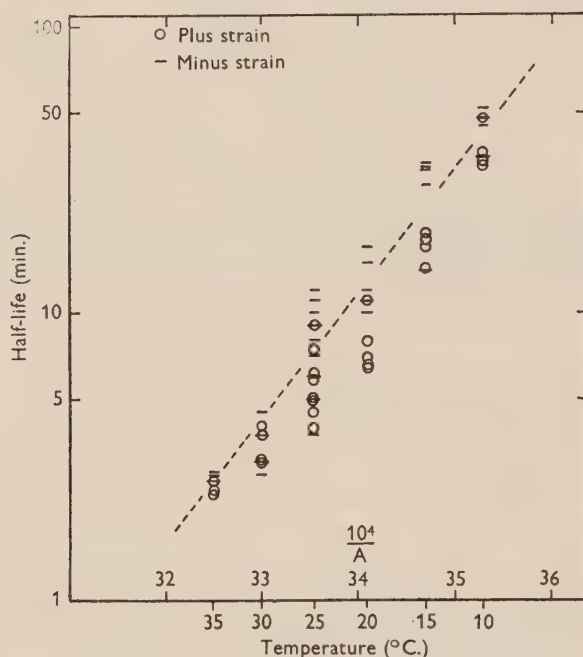


Fig. 19. Half-life values for decay of sexual activity in darkness. Each point represents a separate experiment, the slope of the decay curve being estimated by eye as in Fig. 4.

probability of occurrence. A system of this sort is comparable with that of molecules in a first-order reaction, which react at a rate proportional to their concentration at any moment. From a plot of the logarithms of the half-life values (Table 1) against the reciprocals of the absolute temperatures (Fig. 19), the activation energy for this event may be estimated to lie in the region of 20 Kcal. per particle. (The Arrhenius theory on which this is based is discussed by Johnson, Eyring & Polissar, 1954). This value is of doubtful significance, however, since such a one-particle mechanism appears unlikely.

It would seem more reasonable to assume that the sexual activity depends on a hormone concentration which exceeds a certain threshold, and which may vary somewhat according to the age and physical condition of each organism. If the values of concentration of hormone/organism are distributed about a mean, then as the mean value falls the number of active gametes is



diminished. In the presence of an excess of potential mates, the number of pairs formed can be taken as a measure of the number of active gametes in which the hormone level exceeds this threshold.

These arguments are based on the hypothesis that at any one time the organisms in a suspension can be classified as sexually active or inactive. A further possibility should be considered, namely, that the individual organisms in a suspension may be more or less active. We may suggest, for instance, that in active suspensions a considerable fraction of the length of each flagellum is 'sticky', so that a high proportion of contacts between *plus* and *minus* flagella result in adhesion and ultimately in pairing. A decrease of sexual activity may then result from a decrease in the number of adhesive sites on the flagella, perhaps by the dissolution of some adhesive agent into the medium, and a consequent decrease in the frequency of fruitful contacts. Alternatively, this frequency may be controlled in some way by the concentration of a hormone within each organism. The present experimental data are not adequate to test the relative validities of these two hypotheses—whether sexual activity is an all-or-none phenomenon or not.

The fact that in darkness sexual activity is lost more rapidly under anaerobic conditions than in the presence of oxygen (Fig. 4) can be explained tentatively in at least two ways: (a) The inactivation of the hormone is a fermentative process, perhaps tied up with dark fermentations such as those reported in this species by Frenkel (1952). (b) Hormone formation is an endergonic process which proceeds rapidly in light, but which is capable of continuing slowly even in darkness, driven by the energy of endogenous respiration. However, since the rate of decay far exceeds that of synthesis, there is a net loss of mating activity in darkness, the rate of which is accelerated when respiration is arrested in anaerobiosis.

The decay of mating activity is delayed in organisms which have received some hours of illumination before being placed in darkness. This 'dark lag' period also may be explained in two ways. (a) We may consider that maximum pairing is achieved when the amount of hormone in all potential gametes reaches or exceeds the threshold level. Further illumination produces an excess, which is stored; and when such organisms are placed in darkness they retain full mating activity until the reserve of hormone has become depleted to a level below the threshold value. (b) We may suppose that mating is controlled by a photolabile inhibitor, which is accumulated when organisms are incubated in darkness. In this case, one would expect prolonged incubation to lead to the formation of excess inhibitor, which would presumably extend the lag period of the photoactivation curve. The fact that the 'light lag' appears to be unaffected by the period of prior incubation in darkness (Fig. 11) argues against this possibility.

It will be noted that, whereas the half-life values obtained for *plus* and *minus* strains are closely comparable, the duration of the dark lag tends to be considerably longer in *minus* than in *plus* suspensions under similar conditions. Förster & Wiese (1954*a, b*) indicated somewhat similar behaviour in the female strain of *Chlamydomonas eugametos*, which is sexually equivalent to, if not

identical with, the *minus* strain of *C. moewusii* (see Lewin, 1954). This difference in behaviour between the two mating-types may be taken to indicate that, in a given time, *minus* gametes are capable of accumulating mating hormone more rapidly than are *plus* gametes. Correlated with this, perhaps, are the observations that the light threshold for photoactivation at 25° is lower in *minus* than in *plus* organisms, and that at 300 f.c. photoactivation occurs over a wider temperature range in *minus* organisms than in *plus*. These differences are not necessarily intrinsically bound up with mating-type differentiation, however. The two strains of *C. moewusii* may fortuitously possess many minor genetic dissimilarities, and further study would be needed to reveal whether any such difference is sex-linked or not.

The evidence from the action spectrum and from the effects of phenylurethan certainly points to some close interrelation between sexual activation and photosynthesis. A comparison between the intensities required for light-saturation of the two processes would provide further evidence on this point. A clue to the factor controlling sexual activity was sought in the work of Calvin *et al.* and of Fager *et al.* (reviewed by Gaffron, Fager & Rosenberg, 1951). These workers, in the course of studies on photosynthesis using radioactive  $^{14}\text{CO}_2$ , adduced evidence for the existence of an unstable  $\text{CO}_2$ -acceptor, accumulated by *Scenedesmus* cells during a period of pre-illumination, and rapidly decaying in darkness. C. Ouellet & M. Lefrançois (personal communication), using similar techniques, have calculated the half-life of the  $\text{CO}_2$ -acceptor in *Chlamydomonas moewusii* (*minus* strain) to be *c.* 1.5 min. at 20°. At this temperature, the half-life for the loss of mating activity in darkness is about 15 min., indicating that the two processes are unrelated. Moreover, there is no evidence that after prolonged illumination the  $\text{CO}_2$ -acceptor exhibits any such lag period, prior to the onset of decay, as is exhibited in the case of mating activity. Finally, it has been shown that mating activity can be restored to inactive organisms by illumination in the absence of  $\text{CO}_2$  (other than that metabolically produced *in situ*), which would seem to preclude the possibility that sexual activity is controlled by the accumulation of a photosynthesized product.

This, however, does appear to be the case in certain other species of *Chlamydomonas*. Sager & Granick (1954) showed that *C. reinhardi* organisms may be sexually active in light or darkness provided they are suspended in a medium deficient in assimilable nitrogen. Sexual activity is lost in nitrogenous media; but it may be regained when the organisms are illuminated and when, as a consequence of photosynthesis and assimilation, the medium becomes depleted of nitrogen sources. Tsubo (1956) has obtained similar results with another species, provisionally designated as *Chlamydomonas* sp. 24. In *C. moewusii*, on the other hand, sexual activity is restored by light, and maintained, in media containing ammonium or nitrate salts (0.03M  $\text{NaNO}_3$ ,  $\text{NH}_4\text{Cl}$ ,  $\text{NH}_4\text{NO}_3$  or  $\text{Ca}(\text{NO}_3)_2$ ) just as well as in N-free buffer (FM). It is to be hoped that the 400-odd known species of *Chlamydomonas* have not all evolved different mechanisms in which sexual activity may be controlled by light, and that further work will help to bring the several approaches discussed above into a more unified picture.







LEWIN, R. A.—LIGHT AND SEXUALITY IN *CHLAMYDOMONAS* SP. PLATE 1

(Facing p. 185)

Among the many colleagues and friends who have offered valuable suggestions in the course of this work and in the preparation of this paper, Dr C. Woese of Yale University and Dr C. R. Masson of the N.R.C. Laboratory in Halifax have been especially helpful and stimulating. Miss B. J. Turner and Mrs J. Menzies have assisted in the tedious task of pair-counting, employing reaction chambers constructed by Mr G. Ensell of N.R.C. Ottawa. The earlier phases of this study were carried out at Yale with the financial assistance of a Seissal Post-doctoral Fellowship. The bulk of the work was effected at the Maritime Regional Laboratory of the National Research Council of Canada. This paper is issued as N.R.C. No. 4003.

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## EXPLANATION OF PLATE

Photograph of apparatus employed. Organisms are grown in flasks (1, 2). Samples of 'tester' suspension are maintained in tubes in a perspex holder (3) under constant illumination (4). A reaction chamber, similar to that shown dismantled (5), is enclosed in the constant-temperature tank (6). The water in this is cooled by running water (7) from a refrigerated tank (not shown), heated by an immersion heater (8) connected with a thermostat (9) and relay (10), and equipped with a stirrer (11). Organisms to be tested are drawn into the chamber through the capillary (12) by suction from an aspirator (13). At intervals, samples are expelled through the delivery capillary (12) by pressure on the syringe (14), and are collected in a tube of tester suspension. After being shaken, the mixture is at once darkened in the opaque wooden holder (15) and covered by an aluminium cap (16). When required, illumination of the chamber is provided by an incandescent lamp (17) connected to a rheostat (18). Light passes through an iris diaphragm (19) and is focused by a lens (20) through the perspex window (21) in the tank. Otherwise, light can be completely excluded from the interior of the tank by the light-tight lid (22) and an opaque panel covering the window (21). (Photograph F. Mason.)

(Received 6 February 1956)

## Iron and the Synthesis of Cytochrome $c_3$

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**SUMMARY:** *Desulphovibrio desulphuricans* (El Agheila Z), in lactate + sulphate media, required 10  $\mu\text{mole}$  Fe/ml. for optimal growth; no absolute iron requirement was demonstrable in pyruvate + sulphate media but iron up to 20  $\mu\text{mole}$ /ml. stimulated growth. In a sulphate-deficient pyruvate medium, in which this strain can grow, iron was not required nor did it stimulate growth. Organisms grown with limited iron formed limited amounts of cytochrome  $c_3$ , and in the absence of iron were markedly deficient in this; their content of hydrogenase was reduced to a lesser extent and their content of desulphoviridin was only halved. Iron-deficient organisms were able to reduce sulphate only slowly. Less than 1 % of the iron added to cultures was accounted for as cytochrome  $c_3$ , though only some 6 % of this iron remained in solution after growth.

The demonstration of cytochrome  $c_3$  as a component of the anaerobic sulphate-reducing bacteria (see Postgate, 1956, for references) provided at least a partial explanation of their requirement for traces of iron (Butlin, Adams & Thomas, 1949). The present paper reports a study of the effect of iron on the synthesis of cytochrome  $c_3$ , desulphoviridin (the porphyrin-like pigment which absorbs at 630  $\mu\mu$ ), and of hydrogenase by a strain of these bacteria chosen because of its ability to grow without sulphate when pyruvate is present (Postgate, 1952).

### METHODS

**Organism.** *Desulphovibrio desulphuricans* strain El Agheila Z (National Collection of Industrial Bacteria, NCIB, no. 8380) was a variant, containing hydrogenase, of the organism isolated from a sulphur-producing lake in Libya (Adams, Butlin, Hollands & Postgate, 1951). It was subcultured weekly under  $\text{H}_2$  95 % (v/v)  $\text{CO}_2$  5 % (v/v) at 30° in LSY medium (see below); its purity was checked as described by Postgate (1953).

**Media.** Medium LSY consisted mainly of lactate, sulphate and yeast extract; it was similar to medium C of Butlin *et al.* (1949) but was supplemented with 2.5 % NaCl. Medium PY was a sulphate-deficient medium containing pyruvate and yeast extract (Postgate, 1952). Medium PSY was a similar medium supplemented with  $\text{Na}_2\text{SO}_4$  (18  $\mu\text{mole}$ /ml.).

**Inocula.** For routine subculture 0.05 ml. of parent culture was added to 5 ml. fresh medium. For growth tests in media of defined iron concentration a smaller inoculum was used to avoid carry-over; the parent culture was diluted *c.* 1/2500 to give 0.1  $\mu\text{g}$ . dry wt. organisms/ml. subculture (*c.*  $4 \times 10^5$  viable bacteria/ml.), the concentrations of medium components being adjusted so that the final volume after all additions was 5 ml.;  $\text{Na}_2\text{S}$  (5  $\mu\text{mole}$ /ml.) was included in all media to adjust the redox potential to *c.* -200 mv. and thus to ensure growth of this relatively small inoculum (see Grossman & Postgate, 1953).



*Iron-free reagents and glassware.* Media and solutions to be added were prepared with analytical grade reagents and freed from iron with 8-hydroxyquinoline (Waring & Werkman, 1942), except that  $\text{Na}_2\text{S}$  solutions were assumed to be effectively iron-free owing to the low solubility product of  $\text{FeS}$ . Chromic acid-washed Pyrex glassware was freed from adsorbed iron as recommended by Waring & Werkman; all-glass Seitz filters, used for sterilizing  $\text{Na}_2\text{S}$  solutions and media PY and PSY, were freed from adsorbed iron by washing successively with chromic acid, distilled water, a solution of 8-hydroxyquinoline in water saturated with chloroform, chloroform, and iron-free distilled water.

*Culture in media of defined iron concentration.* Cotton-wool plugs are unsuitable for use with iron-deficient media because of their high iron content. On the other hand, aluminium or glass-capped test-tubes are not entirely suitable for use with cultures of exacting anaerobes in an anaerobic jar, since the gas phase in the jar cannot be changed without the risk of sweeping contaminant micro-organisms under the caps as the new gas phase enters the evacuated jar. This procedure was nevertheless adopted, cultures in test-tubes with aluminium caps being stood in McIntosh & Fildes anaerobic jars on filter-paper wetted with dilute  $\text{CuSO}_4$  solution; cultures were frequently examined microscopically for contaminants, and doubtful ones tested as described by Postgate (1953). In fact, only one culture out of 300 or 400 used in this work contained a contaminant. Duplicate cultures were set up at all iron concentrations, and crucial tests were repeated three times.

Larger quantities of bacteria were grown in 'iron-free' Pyrex glass flasks with beakers as caps, or, when *c.* 2 l. of culture was required, in 'iron-free' flasks with cotton-wool plugs protected with washed gauze. The use of  $\text{Na}_2\text{S}$  as a supplement to the test media ensured growth of the relatively small inocula necessary to eliminate carry-over of iron, but it had the disadvantage that iron could not conveniently be tested at concentrations above 20  $\text{m}\mu\text{mole/ml.}$  because at this concentration turbidity due to  $\text{FeS}$  appeared on addition of  $\text{Na}_2\text{S}$ . This observation supports Seidell (1940), who quoted a value of 70.1  $\text{m}\mu\text{mole/ml.}$  for the saturation solubility of  $\text{FeS}$  at  $18^\circ$  but stated that this figure was probably too high.

*Spectrophotometry.* The application of Barer's (1955) procedure to the spectrophotometry of intact *Desulphovibrio desulphuricans* is described elsewhere (Postgate, 1956). The cytochrome  $c_3$  content of the bacteria was measured at 554  $\text{m}\mu.$ , since, though the Soret peak at 419  $\text{m}\mu.$  would have been a far more sensitive index of  $c_3$  content, the green protein desulphoviridin absorbs at 411  $\text{m}\mu.$  and would be expected to interfere at low concentrations of  $c_3$ . Organisms suspended in strong serum albumin solution were reduced with  $\text{Na}_2\text{S}_2\text{O}_4$  in 0.5 cm. optical cells and the absorption relative to reduced controls was determined at 554  $\text{m}\mu.$ ; the specific extinction coefficient of cytochrome  $c_3$  was taken as 4.2 and the absorption due to  $c_3$  calculated from a hypothetical linear scatter curve using the arithmetical extrapolation below ( $d$ =cell density in mg. dry wt./ml.):

$$\text{mg. } c_3/\text{g. air-dry wt.} = \frac{2}{4.2 \times 10^{-3}d} \left[ \epsilon_{554} - \left( \epsilon_{570} + \frac{\epsilon_{538} - \epsilon_{570}}{2} \right) \right].$$

Several spectra of intact bacteria in the 500–600 m $\mu$ . range showed that this expression could legitimately be used provided the absolute optical density of the test suspension was below 2.0.

Cytochrome  $c_3$  contents of bacteria are also quoted as  $\mu$ -units ( $\mu$ U.)/g.; 1  $\mu$ U. would equal 1  $\mu$ mole if the millimolar extinction coefficient were 54, the value to be expected for a bifunctional haematohaematin.

Desulphoviridin contents of bacteria were estimated by plotting the spectrum of intact organisms between 600 and 660 m $\mu$ . and estimating the height of the inflexion at 630 m $\mu$ .; they are expressed in arbitrary units: optical density at 630 m $\mu$ . /g. dry wt. bacteria.

*Hydrogenase activity.* The hydrogenase content of the organisms was judged by the rate of reduction of methylene blue: 2.6–3 mg. dry wt. organisms in buffer (KH<sub>2</sub>PO<sub>4</sub> 0.5 %, w/v, pH 6.30  $\pm$  0.02, + NaCl 2.5 %, w/v) were incubated in conventional Warburg manometers under H<sub>2</sub> at 37° for at least 1 hr. before tipping in 7.5  $\mu$ mole substrate; this pre-incubation was necessary to obtain maximum reduction rates.

*Sulphide estimation.* Cultures for sulphide determination were grown *in vacuo* in sealed 'iron-free' Pyrex tubes. Immediately after opening them, sulphide was determined iodometrically as described by Grossman & Postgate (1955), applying their correction: iodine equivalent to 2.2  $\mu$ mole S''/ml. absorbed by 1 mg. dry wt. bacteria/ml.

*Bacterial densities.* These were measured turbidimetrically in the E.E.L. colorimeter (Evans Electroselenium Ltd.) calibrated with the Hildenborough strain of these bacteria.

## RESULTS

*Iron requirement of Desulphovibrio desulphuricans, Strain El Agheila Z.* This strain required 10  $\mu$ mole Fe/ml. for optimal growth in medium LSY (Fig. 1). Little iron remained after growth with a suboptimal iron concentration. The supernatant fluid from a 5-day culture in medium LSY + 7.5  $\mu$ mole Fe/ml. was acidified, concentrated tenfold and analysed for iron with o-phenanthroline (Sandell, 1944); it contained only 0.48  $\mu$ mole Fe/ml., equivalent to 6.4 % of the amount added initially.

Strain El Agheila Z had no demonstrable *requirement* for iron in medium PSY, but iron up to 20  $\mu$ mole/ml. stimulated growth; growth was not linearly related to iron concentration. In medium PY the organism had again no requirement for added iron, and iron up to 20  $\mu$ mole/ml. had little or no effect on growth (Fig. 1).

*Sulphate reduction by iron-deficient cells.* The bacterial densities reached in iron-deficient media PSY and PY were similar, and suggested that, even in the presence of sulphate, only 'sulphate-deficient' growth occurred. The rate of sulphide formation in medium PSY with and without iron was therefore determined. The results are recorded in Table 1: with 20  $\mu$ mole Fe/ml. all the available sulphate was reduced in less than 100 hr.; without Fe only about half was reduced after 550 hr. Though a lower bacterial density was reached in the absence of Fe, this was nevertheless half that reached in its presence and could

not account for the tenfold lowering in rate of sulphide formation. Mucopolysaccharide formation, mentioned by Grossman & Postgate (1955), occurred in both media and rendered determinations of optical density invalid after prolonged incubation. It was formed at an earlier stage in medium PSY than in medium PY; detailed quantitative data were not obtained.

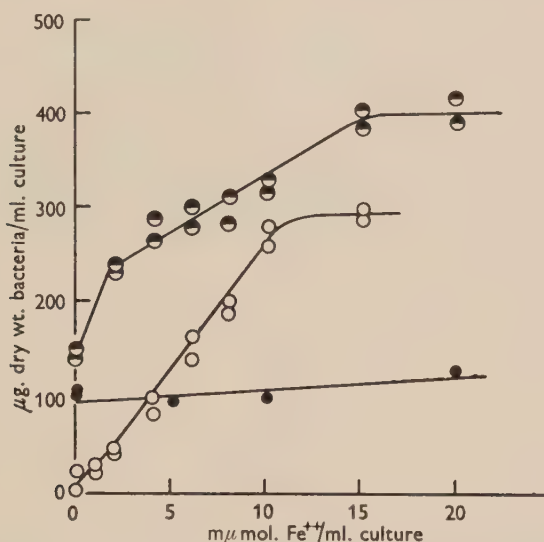


Fig. 1. Effect of iron on growth of *Desulphovibrio desulphuricans* in lactate or pyruvate media. Cultures of *D. desulphuricans* strain El Agheila Z were incubated in lactate or pyruvate media of known iron content ( $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4$  for media PSY and LSY;  $\text{FeCl}_2$  for medium PY), and the amounts of growth determined after 5 days of incubation.  $\circ$  = medium LSY;  $\bullet$  = medium PSY;  $\bullet$  = medium PY.

Table 1. *Sulphide formation by Desulphovibrio desulphuricans (El Agheila Z) in pyruvate medium with and without added iron*

Iron-deficient medium PSY (see text) was distributed in iron-free tubes, inoculated (c.  $3\mu\text{g}$ . dry wt. organisms/ml.), sealed *in vacuo* and incubated at  $30^\circ$ . Pairs of tubes were opened and the amounts of growth and sulphide concentrations were determined at intervals.

Incubation time (hr.)	Without added iron		With $20\text{ m}\mu\text{mol}$ . $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2/\text{ml}$ .	
	Dry wt. organisms $\mu\text{g.}/\text{ml}$ .	Sulphide $\mu\text{mole}/\text{ml}$ .	Dry wt. organisms $\mu\text{g.}/\text{ml}$ .	Sulphide $\mu\text{mole}/\text{ml}$ .
72	48	—	260	5.43
120	90	2.33	295	8.1
144	150	2.18	240	8.85
216	140	2.70	300*	9.2
230	130*	4.62	290*	8.85
360	105*	3.90	185*	9.75
550	c. 50*	4.50	c. 30*	9.25

\* The optical densities of these suspensions were diminished by mucopolysaccharide formation; hence these readings are low.



Iron deficiency decreased the activity of suspensions of bacteria to an even greater extent. Organisms grown in an iron-deficient medium reduced sulphate in the presence of hydrogen at only about one-fiftieth of the rate observed with organisms grown with iron (Table 3). The decline may in fact have been greater than this, since the quoted  $Q$  values imply a net difference between blank and control vessels of 3 to 6 mm.<sup>3</sup> H<sub>2</sub> absorbed over 1½ hr., a difference which is close to the limit of sensitivity of the manometric procedure used. The decline in rate of sulphate reduction could not be due to deficiency in hydrogenase, since there was only a fivefold to eightfold decrease in the hydrogenase activity of iron-deficient organisms (below).

*Effect of iron on content of cytochrome c<sub>3</sub>.* Waring & Werkman (1944) showed that iron-deficient *Aerobacter indologenes* was deficient in cytochrome. The fact that the iron requirement of strain El Agheila Z depended on the test medium permitted a quantitative study of this effect in *Desulphovibrio desulphuricans*. Bacteria grown in medium LSY containing 5, 7.5 or 10 mμmole Fe/ml. contained cytochrome c<sub>3</sub> in concentrations proportional to the iron concentration (Table 2). Organisms harvested from pyruvate media (PY and PSY) contained plentiful cytochrome c<sub>3</sub> when iron was present in the medium; in the absence of iron cytochrome c<sub>3</sub> was present only in very small amounts and was undetectable unless very heavy suspensions were used in the spectrophotometer. Provided iron were present, however, cytochrome c<sub>3</sub> was found both in the presence and absence of sulphate.

Table 2. *Cytochrome c<sub>3</sub> and desulphoviridin contents of Desulphovibrio desulphuricans grown in media of defined iron concentration*

Batches (250 ml. to 1 l.) of *D. desulphuricans* strain El Agheila Z were grown in iron-deficient media with known amounts of added iron (as FeCl<sub>2</sub> or Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>), centrifuged, and the content of cytochrome c<sub>3</sub> and desulphoviridin/g. dry wt. organisms was determined spectrophotometrically (see text).

Medium	Added iron (mμmole/ml.)	cytochrome c <sub>3</sub> content		Desulphoviridin content (optical density value/g.)
		(mg./g.)	(μU./g.)	
LSY	5	1.19	0.091	6.4
LSY	7.5	1.35	0.104	5.1
LSY	10	1.87	0.144	6.1
PY	—	0.121	0.0093	4.0
PY	20	2.3	0.177	8.7
PSY	20	1.83	0.141	7.8
PSY	—	0.0195	0.00153	3.8

*Effect of iron on porphyrin synthesis.* Iron deficiency sometimes leads to accumulation of porphyrin in culture media (see Lascelles, 1955); no porphyrin was detected in cultures of El Agheila Z in iron-free media PSY or PY.

*Effect of iron on content of desulphoviridin.* The amount of this pigment/unit wt. of organisms was little affected by the iron content of the culture medium (Table 2), in contrast to the amount of cytochrome c<sub>3</sub>. This observation is consistent with Postgate's (1956) view that desulphoviridin is a porphyrinoprotein and not a haematin as supposed by Ishimoto, Koyama & Nagai (1954*a, b*).

*Effect of iron on content of hydrogenase.* Waring & Werkman (1944) showed that *Aerobacter indologenes* was deficient in hydrogenase when grown in iron-deficient media; it was of interest to see if this were also true of *Desulphovibrio desulphuricans*. Table 3 lists some  $Q$  values for the reduction of methylene blue in hydrogen and shows that growth without Fe led to a fivefold to eightfold decline in the hydrogenase content.

Table 3. *Effect of iron on the hydrogenase content of Desulphovibrio desulphuricans*

*D. desulphuricans* strain El Agheila Z organisms were harvested from iron-deficient media PY or PSY in 'iron-free' vessels (see text) after incubation for 3–5 days at 30° under  $H_2 + 5\%$   $CO_2$ . The rates of hydrogen absorption with sulphate or methylene blue as electron acceptor were compared with the rates for organisms harvested from similar media but with 15 to 20  $\mu$ mole Fe/ml. added. Vessels were pre-incubated  $1\frac{1}{2}$  hr. before addition of substrates;  $-Q$  values ( $mm^3H_2$  absorbed/mg dry wt. organisms/hr.) quoted from three experiments.

Growth medium	With iron			Without iron		
	$-Q_{H_2}^{MB}$	$-Q_{H_2}^{SO_4''}$	$-Q_{H_2}^{endogenous}$	$-Q_{H_2}^{MB}$	$-Q_{H_2}^{SO_4''}$	$-Q_{H_2}^{endogenous}$
PY	192	—	—	48	—	—
PSY	230	38	4.8	29	3.2	2.6
PSY	95	45	0.5	31	3.1	2.6

#### DISCUSSION

*Recovery of iron as cytochrome  $c_3$ .* The molar extinction coefficient of cytochrome  $c_3$ , together with its relatively high iron content, provide reasons for assuming the presence of two haemin groups per molecule (Postgate, 1955). The recovery of added iron as cytochrome  $c_3$  can therefore be calculated. For example: a culture of El Agheila Z in medium LSY + 5  $\mu$ mole Fe/ml. had a final bacterial density of 250  $\mu$ g. dry wt./ml. and the organisms contained 0.091  $\mu$ U.  $c_3$ /g.; hence the cytochrome accounted for 0.9% of the added iron. Similar calculations for El Agheila Z grown with other concentrations of iron gave these values: recovery as  $c_3$  from 7.5  $\mu$ mole Fe/ml.: 0.78%; 10  $\mu$ mole Fe/ml.: 0.88%.

Clearly a large proportion of the added iron was not used for cytochrome  $c_3$  synthesis although over 90% of it was removed from solution. It was not detectable as haematin, since treatment of the whole organisms with NaOH, pyridine and dithionite did not increase the intensity of the  $\alpha$ -band of  $c_3$ , though it shifted 2 or 3  $m\mu$ . towards the violet; the fate of the remainder of the added iron is thus unknown. Higher concentrations of cytochrome  $c_3$  were observed with the Hildenborough strain grown on media of unknown iron content by continuous culture procedures; the organisms used by Postgate (1956) for the preparation of cytochrome  $c_3$  contained about 0.20  $\mu$ U.  $c_3$ /g.

Though the concentration of cytochrome  $c_3$  in the organisms was low compared with the amount of iron added, it was roughly proportional to the amount of iron added and was not influenced by the presence of sulphate even though the cytochrome probably acts as a carrier in sulphate reduction. Thus strain El Agheila Z of *Desulphovibrio desulphuricans* differed markedly from the yeast

studied by Slonimski (1955), which formed cytochrome *c* and many other enzymes of the respiratory cycle, adaptively, in response to the presence of its terminal electron acceptor oxygen.

*Biological function of cytochrome  $c_3$ .* Evidence for the view that cytochrome  $c_3$  acts as a carrier between sulphate reduction and cellular oxidation processes in *Desulphovibrio desulphuricans* has been discussed elsewhere (Ishimoto *et al.* 1954*b*; Postgate, 1956); the present work supports this view in that organisms grown in media freed from iron by 8-hydroxyquinoline are deficient in cytochrome  $c_3$  and can reduce sulphate only slowly. But neither this observation nor earlier evidence demonstrates conclusively that cytochrome  $c_3$  is the natural carrier for the reduction of sulphate. The oxidation of cytochrome  $c_3$ , observed when whole organisms are incubated with sulphate, may only reflect a general change in the redox potential of organisms which are metabolizing sulphate. The ability of methyl and benzyl viologen to substitute for cytochrome  $c_3$  in the reduction of sulphite, thiosulphate, etc., by enzymically active cell preparations suggests that the carrier action of  $c_3$  may be merely a reflexion of its low redox potential and have no real metabolic significance. The fact that cytochrome  $c_3$  acts as a carrier in the hydrogen-oxygen reaction, which can scarcely be of great metabolic importance for so strict an anaerobe as *Desulphovibrio desulphuricans*, does not strengthen confidence in the view that it is the biological carrier with other terminal electron acceptors.

I am grateful to Dr P. Slonimski for a valuable discussion of this work during its early stages. I am also indebted to Mr George Coldicott for preparing basal media and iron-free glassware, and to my colleagues of the Microbiology Group of this Laboratory for interest and encouragement. This paper is published by permission of the Director, Chemical Research Laboratory.

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## The Effect of a Physical Barrier on Sporulation of *Chaetomium globosum*

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**SUMMARY:** Increased production of perithecia by *Chaetomium globosum* in the neighbourhood of a physical barrier which impedes the growth of the colony is associated with a locally increased concentration in the medium of organic phosphates excreted by the organism, caused by unequal diffusion of these compounds owing to the presence of the barrier.

It is well known that sporulation of fungi growing in culture media may be stimulated when the mycelium approaches a physical barrier, e.g. the edge of a Petri dish or a channel cut in an agar medium. The subject was studied by Robinson (1926) but no satisfactory explanation of the effect seems yet to have been given. Evidence has accumulated recently that the fruiting of some fungi is stimulated by the presence in the medium of traces of certain organic phosphates (Hawker, 1948; Buston & King, 1951). Buston & Khan (1956) showed that some micro-organisms excrete organic phosphates into the medium, and it seemed possible therefore that sporulation might be stimulated by the presence in the medium of organic phosphate excreted by the organism itself; if free diffusion were hindered by the presence of a physical barrier, phosphate might accumulate locally in sufficient concentration to increase sporulation near that point. In order to test the validity of this suggestion the experiments now described were performed.

### RESULTS

The strain of *Chaetomium globosum* was the stock culture maintained in the laboratory; the cultural methods used were those described by Buston & King (1951).

#### *Production by Chaetomium globosum of a diffusible substance capable of stimulating formation of perithecia in an adjacent colony of the same organism.*

Buston & Basu (1948) obtained indications that a staled medium in which *Chaetomium globosum* had grown slightly stimulated production of perithecia in a young colony; a more direct test was made as follows. An inoculum of *C. globosum* was made 50 mm. from one side of a Petri dish (140 mm. diam.); after incubation at 30° for 48 hr. a second inoculum was made, 30 mm. from the first towards the centre of the dish, and the incubation was continued. In the first colony immature perithecia were evenly distributed in the mycelium within 108 hr. The first perithecia in the second colony were visible 84 hr. after

inoculation, and were at first localized in that part of the mycelium nearest the original colony; others appeared in the remainder of the colony about 24 hr. later. Perithecial counts were made on the 10th day in four quadrants of the second colony, and the results are shown diagrammatically in Fig. 1. The quadrant (*a*) nearest the first colony was distinctly flattened towards the edge, due presumably to the presence of some inhibitory or staling substance produced by the first colony, but production of perithecia was markedly greater than in the other quadrants.

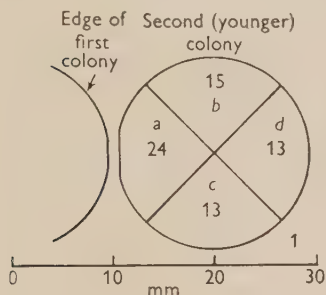


Fig. 1.

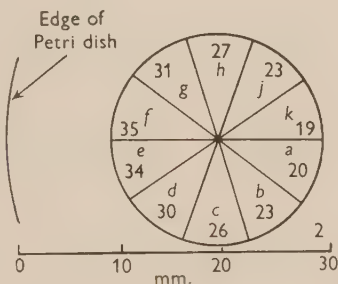


Fig. 2.

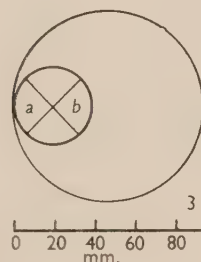


Fig. 3.

Fig. 1. Stimulation of production of perithecia in a colony of *Chaetomium globosum* in the neighbourhood of another colony of the same organism. The figures in quadrants represent perithecial frequency (average of 10 fields/quadrant in each of 3 plates).

Fig. 2. Stimulation of production of perithecia in a colony of *Chaetomium globosum* near the edge of the Petri dish. Figures represent perithecial frequency in sectors (average of 3 fields/sector in each of 10 plates).

Fig. 3. Diagrammatic representation of areas in which distribution of organic phosphate was estimated. Inoculum at centre of inner circle.

#### *Increased formation of perithecia near edge of Petri dish*

Petri dishes 90 mm. diameter were used, and inocula of *Chaetomium globosum* were placed at distances of 10, 20, 30 and 40 mm. from the edge. Perithecial counts were made on the 10th day, the colonies being divided into ten equal sectors for this purpose. As shown in Fig. 2, which shows the results obtained at 20 mm., a significantly increased production of perithecia was observed in the sectors (*e* and *f*) nearest the edge of the dish. When the inoculum was placed 10 mm. from the edge a similar result was obtained but the colony was much flattened towards the edge and the greatest accumulation of perithecia was slightly to each side of the point nearest the edge (i.e. towards sectors *d* and *g*). Maximum perithecial frequencies were of the same order as at 20 mm. When the inocula were placed at distances of 30 mm. or more from the edge, perithecia were evenly distributed throughout the mycelium.

#### *Accumulation of organic phosphates in areas near the colony*

The nature of the organic phosphates excreted by *Chaetomium globosum* was investigated chromatographically as described by Buston & Khan (1956). Glucose-6-phosphate, fructose-1,6-diphosphate, and 3-phosphoglyceric



acid were identified; a small amount of another unidentified phosphate was detected. In order to follow changes in distribution of organic phosphates in different zones of the medium during the growth period, some 60 cultures were prepared, the inoculum in each being placed 20 mm. from the edge of the dish. From the first to the tenth day at least three dishes were taken daily and from each, segments of equal size of the medium were removed: (a) between the point of inoculation and the nearest edge of the Petri dish; (b) from a position symmetrically opposite to this (Fig. 3). Mycelial growth was removed, after which the organic phosphates were extracted from the agar and separated

Table 1. *Relative amounts of organic phosphates present in different parts of the medium during growth of Chaetomium globosum*

Immature perithecia in (a) at 84 hr., in (b) at 108 hr.; mature perithecia in (a) and (b) at 156 hr. G-P = Glucose-6-phosphate; F-P = fructose-1-6-diphosphate; P-G-A = 3-phosphoglyceric acid.

Time after inoculation (hr.)	(a) Sector near edge of dish			(b) Sector furthest from edge of dish		
	G-P	F-P	P-G-A	G-P	F-P	P-G-A
	Relative amounts of organic phosphates					
24	0	0	0	0	0	0
48	2	1	tr	0	0	0
72	4	3	3	0	1	1
96	5	5	6	4	4	tr
120	2	4	>6	3	3½	1
144	0	tr	1	0	tr	4½
168	0	0	tr	0	0	1
192	0	0	0	0	0	0

chromatographically. The technique of extraction, etc., was standardized so that it was possible to obtain a comparative measure of the amounts of the different phosphates on the chromatogram by using an E.E.L. photoelectric 'scanner'; it was not considered necessary to attempt any determination of absolute quantities since the point at issue was to estimate relative concentrations on opposite sides of the colony. From the results obtained (Table 1) it was evident that organic phosphates accumulated more rapidly and reached a greater concentration near the edge of the Petri dish than on the 'open' side of the colony. In both positions glucose-6-phosphate and fructose-1,6-diphosphate were detected before 3-phosphoglyceric acid, which, however, persisted in the medium after the disappearance of the other esters. The rapid decrease in the amount of organic phosphate in the medium at the fifth or sixth day was striking; it coincided approximately with the appearance of mature perithecia, but whether this was more than coincidence remains to be seen. A similar series of experiments in which the inocula were placed at 15 mm. from the edge gave virtually the same results except that maximum concentration of phosphates near the edge was reached some 12 hr. earlier.

## DISCUSSION

The observations now recorded are in agreement with the following suggestions: (1) the appearance of perithecia is associated with the presence of a mixture of organic phosphates in the surrounding medium; these compounds are produced within the mycelium and diffuse outwards; (2) the distribution of perithecia is such as might be expected if free diffusion of the excreted phosphates were impeded by a physical barrier. The intensity of fruiting would appear to be influenced by the state of maturity of the colony and by the distance between the colony and the barrier, these factors together resulting, in favourable circumstances, in a local increase in the concentration of phosphates sufficient to stimulate sporulation.

The experiments here described do not themselves give rigid proof that organic phosphates alone were responsible for the effects noted; their presence is unquestionably associated with production of perithecia but some other substance(s) may have contributed to the stimulatory effect. The local inhibition of growth illustrated diagrammatically in Fig. 1 shows that other active substances are present in the medium, but the well established fact that hexose phosphates in small concentrations can stimulate fruiting affords evidence that they may be directly concerned in the present instance. Previous work (e.g. Buston, Jabbar & Etheridge, 1953) indicated that the maximum effect is produced when the concentration of phosphate lies within certain rather narrow limits and it is of interest to note that in cultures in which the inoculum was only 10 mm. from the edge of the dish most of the perithecia were formed slightly to left and right of the point nearest the edge, i.e. in sectors *d* and *g* rather than *e* and *f* (Fig. 2), suggesting that the concentration of organic phosphate in the latter had exceeded the optimum. Where the inoculum was 30 mm. or more from the edge there was less cause for unequal diffusion and a local concentration of phosphates sufficient to affect sporulation did not result.

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## A Substitute for Bile Salts in Culture Media

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**SUMMARY:** Teepol, a modern anionic detergent, has been investigated and used in culture media for organisms of the coli-typhoid group, in substitution for bile salts. Media containing 0.1% Teepol and an indicator of the sulphonphthalein group have been found to possess advantages over MacConkey's medium. While bile salts as marketed are costly and variable in their bacteriological properties, Teepol is cheap and reliable.

MacConkey (1908) described a culture medium which still has a wide field of use. This medium incorporates an intestinal detergent, sodium taurocholate, which confers selectivity and suppresses the swarming of *Proteus*. Lominski & Lendrum (1942) showed that swarming is inhibited by surface active agents, particularly straight chain alkyl sulphates which have a peak of anti-swarming activity when the molecular chain has 12 carbon atoms.

Several household detergents are known to be bacteriostatic to Gram-positive organisms as well as being surface active, and the possibility of finding one which could replace sodium taurocholate logically presented itself.

### METHODS

The *selectivity factor* of detergents was determined as follows: Duplicate doubling dilutions of known strength in distilled water were prepared. To 0.5 ml. of each dilution of detergent in a sterile tube was added an equal volume of double strength Lemco broth. To each tube of one series was added 0.02 ml. of a 1/2500 dilution in broth of a 6 hr. broth culture of the standard Oxford staphylococcus. A similar inoculum of *Escherichia coli* O 55 was added to the second series. All tubes were examined for turbidity after 24 hr. at 37°; the lowest concentration of detergent showing absence of turbidity was recorded.

The *anti-swarming* factor was determined by pouring plates of doubling dilutions of the detergents in nutrient agar. After drying, each plate was touched at one point with an inoculum of a swarming strain of *Proteus*, and incubated at 37° for 18 hr. The lowest concentration which suppressed swarming was recorded.

We first selected two detergents in laboratory use, Cetrimide (Cetavlon, I.C.I.) for its known bacteriostatic properties, and Teepol (Shell) partly because previous unpublished work had suggested that this detergent might well have a use in culture media, and partly because we knew that it contained alkyl sulphates. The selectivity and anti-swarming factors of these agents were ascertained and compared with those of the following bile salts: sodium taurocholate, sodium tauro-glycocholate and sodium deoxycholate (Table 1). *Escherichia coli* O 55 grew freely in the highest concentrations of



sodium taurocholate and of sodium deoxycholate that were technically convenient to prepare. We have therefore expressed the selectivity and anti-swarming factors of these salts as 'greater than' values. As it would not be practicable to use bile salts in higher concentrations than those at which we tested them, for practical purposes the 'greater than' symbols in the table can be ignored. The results shown in Table 1 indicated that Teepol is potentially a more suitable ingredient for MacConkey's medium than either sodium taurocholate or sodium tauro-glycocholate. Cetrinide also showed potentialities in this direction, but we discarded it in favour of Teepol on account of the relatively low value of the anti-swarming factor of Cetrinide, namely 10.

Before carrying out further tests on Teepol we wished to be satisfied that it is of constant chemical composition. In correspondence with the makers (Shell Chemicals Ltd., Norman House, 105/109 Strand, London, W.C. 2), we were supplied with the following information; the active organic detergent in Teepol consists of sodium or potassium salts of alkyl sulphates, the alkyl radicles ranging from 8 to 18 carbon atom chains. These alkyl groups are present in approximately equal proportions, so that the proportion of 11, 12 and 13 carbon atom chains would be three-elevenths of the total. The method of manufacture is such that the variation from this figure would be very small and probably not more than  $\pm 1\%$ . The other constituents of Teepol as marketed are sodium and potassium sulphates up to 4% by weight, and small residues of unsulphated organic matter which do not exceed 1% by weight.

At our request the makers sent us ten representative batches of Teepol and a sample of the olefines from which it is manufactured, which are liable to be present in the unsulphated organic matter referred to above.

Eight batches of Teepol sent by the makers and two batches in use in the laboratory were tested. The selectivity and anti-swarming factors of each batch were ascertained. These factors were identical with those given in Table 1 in every case, and the end-points obtained in determining these ratios were all the same.

Table 1. *Assay of detergents*

Detergent	<i>Escherichia coli</i> minimum inhibitory concentration (A)	Oxford staphylo- coccus minimum inhibitory concentration (B)	Minimum anti- swarming concentration (C)	Selectivity factor $\left(\frac{1}{B} \div \frac{1}{A}\right)$	Anti- swarming factor $\left(\frac{1}{C} \div \frac{1}{A}\right)$
Sodium taurocholate	$\frac{>1}{12}$	$\frac{1}{50}$	$\frac{1}{400}$	$>4$	$>33$
Sodium tauro- glycocholate	$\frac{1}{12}$	$\frac{1}{400}$	$\frac{1}{400}$	33	33
Sodium deoxycholate	$\frac{>1}{12}$	$\frac{1}{3200}$	$\frac{1}{800}$	$>267$	$>67$
Cetrinide	$\frac{1}{3200}$	$\frac{1}{2,560,000}$	$\frac{1}{32,000}$	800	10
Teepol	$\frac{1}{3}$	$\frac{1}{1600}$	$\frac{1}{3200}$	533	1067

To ascertain whether the sample of olefines had any bacteriologically undesirable property, we attempted to dissolve this material in nutrient broth at 37°. The olefine had an extremely low solubility in broth and a 'saturated solution' of it in nutrient broth at 37° freely supported growth of *Escherichia coli* O55 and of the Oxford staphylococcus.

### *Teepol media*

We decided to use Teepol in a concentration of 0.1 % in media. The results of our tests had indicated that Teepol might safely be used in a much higher concentration than this, but by selecting a concentration near to the minimum effective level we gained the advantage of obtaining a greater dilution of such impurities as might be present. A MacConkey agar was therefore made up containing 0.1 % Teepol in substitution of bile salt. This medium tended to precipitate the neutral red dye. Attention was then directed to the sulphon-phthalein group of indicators. After trial we chose bromthymol blue for solid media. Colonies of *Escherichia coli* are large and opaque, and with this indicator give a characteristic cream colour. Colonies of *Salmonella* and *Shigella* are less opaque and are pale green except in the vicinity of lactose-fermenting colonies when they show up as a deeper shade of yellow against the creamy colonies of *E. coli*. Bromcresol purple was found to give a more clear-cut result in liquid media since with this indicator the colour change does not begin to occur until the pH has fallen to 6.8.

The composition of the media used in our laboratory trials was as follows:

Teepol agar	Lemco agar	MacConkey agar
2 % Eupeptone No. 2 (Allen and Hanburys)	1 % Eupeptone No. 2 (Allen and Hanburys)	2 % Eupeptone No. 2 (Allen and Hanburys)
1 % Lactose	1 % Lab. Lemco	1 % Lactose
0.5 % Sodium chloride	0.5 % Sodium chloride	0.5 % Sodium chloride
0.1 % Teepol		0.5 % Sodium taurocholate
0.005 % Bromthymol blue		0.003 % Neutral red

All media were solidified with 0.9 % New Zealand (Davis) agar, and the pH value adjusted to 7.6. The composition of the fluid media was identical with that of the respective solid media except for the exclusion of agar from the former, the use of 0.005 % bromcresol purple in both fluid media, and the substitution of sodium tauro-glycocholate for sodium taurocholate in MacConkey broth. The pH value of the fluid media was adjusted to 7.5. Since completion of the trials we have preferred the use of bromcresol purple at 0.001 %.

### RESULTS

*Teepol agar.* In order to show that Teepol agar is as favourable to intestinal pathogens as MacConkey agar, plate counts were made by the technique of Miles & Misra (1938) using six tenfold dilutions on each of three plates. The counts were put up in quadruplicate using the following media: Lemco agar, MacConkey agar containing 0.5 % sodium taurocholate (taurocholate MacConkey), MacConkey agar containing 0.5 % sodium tauro-glycocholate (tauro-

glycocholate MacConkey), and Teepol agar. Counts were made on broth cultures of ten intestinal pathogens and of two non-pathogens. The results of these counts, expressed in millions, are set out in Table 2, from which it will be seen that in eleven instances out of twelve the counts on Teepol agar were higher than on taurocholate MacConkey agar. The counts on Lemco agar, tauro-glycocholate MacConkey and Teepol agar did not differ from each other significantly.

Table 2. Plate counts made from 12 organisms on Teepol agar, Lemco agar and on two bile salt media

Counts are expressed in millions of organisms/ml.

Organisms	Lemco agar	Taurocholate MacConkey agar	Tauro- glycocholate MacConkey agar	Teepol agar
<i>Salmonella typhi</i>	300	265	340	320
<i>S. paratyphi-B</i>	335	305	350	350
<i>S. typhimurium</i>	320	350	315	355
<i>Shigella sonnei</i>	320	235	355	360
<i>S. flexneri</i> type 2	250	255	230	235
<i>Escherichia coli</i>				
O 26	445	345	395	380
O 55	425	370	435	400
O 111	290	250	305	305
O 119	350	315	385	325
O 128	365	305	370	335
<i>Citrobacter freundii</i>	250	165	210	260
<i>Aerobacter aerogenes</i>	700	500	750	680

Colony diameters of six organisms were measured. Nine discrete colonies of each were measured on each of the four media, three colonies being measured on each of the three plates in the sector giving the smallest number of colonies greater than 2. Measurements were made by use of an eyepiece micrometer and a plate microscope, and were recorded as means of nine measurements. These mean values are shown in Table 3. From this table it will be seen that colonies of *Salmonella typhimurium* and of five pathogenic strains of *Escherichia coli* were larger on Teepol agar than on any of the three other media, including nutrient agar.

Table 3. Colony diameters of six organisms on Lemco agar, Teepol agar, and on two bile salt media

Sizes are expressed in millimetres and each is the mean of nine colonies measured.

Organism	Lemco agar	Taurocholate MacConkey agar	Tauro- glycocholate MacConkey agar	Teepol agar
<i>Escherichia coli</i>				
O 26	2.0	1.5	2.1	2.3
O 55	2.0	1.4	2.1	2.6
O 111	2.1	1.4	1.9	2.3
O 119	2.1	1.4	1.9	2.3
O 128	1.9	1.6	1.8	2.2
<i>Salmonella typhimurium</i>	2.1	1.4	1.5	2.3



One or more strains of twelve serotypes each of *Shigella* and of *Salmonella* other than those listed in Table 3 were also found to grow freely on Teepol agar, and no strain of either was inhibited. Enterococci, micrococci and staphylococci did not grow.

A comparison was then made between Teepol agar and MacConkey agar in the isolation of serologically identifiable strains of *Escherichia coli* from rectal swabs taken from babies with gastro-enteritis. In every instance the swab was rubbed first on to the MacConkey agar plate and then on to the Teepol agar plate. In spite of this disadvantage a serologically identifiable strain of *E. coli* was isolated from the Teepol plate in every one of thirty-three instances in which it was also isolated from the MacConkey plate. In sixty-seven instances no isolation was made from either plate.

*Teepol broth* was tested in parallel with MacConkey broth for presumptive coli counts on 166 drinking waters and 162 sea waters. Ten ml. of drinking water were added to 5 tubes, 1.0 ml. to 5 tubes and 0.1 ml. to 5 tubes of each medium. Six tubes only were used for sea waters, 1.0 ml. being added to 2 tubes of each medium, 0.1 ml. to 2 tubes and 0.01 ml. to 2 tubes. Tubes showing acid and gas after 48 hr. at 37° were subcultured to further tubes of the same medium at 44° and read after 24 hr. Counts at 44° were thus made on 112 drinking waters and 143 sea waters. All these counts are summarized in Tables 4 and 5.

Table 4. *Drinking waters*

	Teepol broth	MacConkey broth
(a) Number of tubes set up	2490	2490
(b) Number of presumptive positives (i.e. 37°)	1071	1014
Percentage	43.0	40.7
(c) Number of confirmed positives (i.e. 44°)	539	498
Percentage	21.7	20.0
(c) expressed as a percentage of (b)	50.3	49.1

Table 5. *Sea waters*

	Teepol broth	MacConkey broth
(a) Number of tubes set up	972	972
(b) Number of presumptive positives (i.e. 37°)	521	524
Percentage	53.6	53.9
(c) Number of confirmed positives (i.e. 44°)	312	319
Percentage	32.1	32.8
(c) expressed as a percentage of (b)	59.9	60.9

The mean probable numbers for the 37° and 44° counts on the drinking waters were interpolated by means of Table 4 of the Report (1939). 31.3 % of the 37° counts were higher in Teepol, 43.4 % were equal in the two media, and 25.3 % were higher in MacConkey broth. The corresponding percentages for the 44° counts were 46.4, 33.0 and 20.5 % respectively.

## DISCUSSION

In choosing ingredients for a differential medium for *Bacillus coli communis* and *B. typhi abdominalis* MacConkey adhered to the principle that these organisms should grow best in conditions most nearly identical with their normal habitat, the human intestine. The inclusion of bile salts in his medium was therefore a logical step which has been amply endorsed by the test of time. Teepol and other modern detergents had not been introduced in MacConkey's day, and even if they had been, MacConkey would have had no reason to suspect that they might be more useful in his medium than bile salts.

The tests on MacConkey agar and Teepol agar substantiated the earlier tests which led us to believe that Teepol had properties more favourable for use in culture media than those of sodium taurocholate and sodium tauro-glycocholate. Teepol agar was shown to support the growth of a wide range of intestinal pathogens which grow on MacConkey agar, to produce larger colonies, to suppress the swarming of *Proteus* species, and to be more selective against Gram-positive organisms.

Since May 1955 Teepol agar has been in routine use in our Laboratory in place of MacConkey agar which we formerly used for the isolation of *Escherichia coli* strains from faeces and for subculture of colony picks to sectors of plates. Colonies on Teepol agar do not readily go rough as on some batches of MacConkey agar and antigenically are at least equally suitable for slide-agglutination tests. Teepol withstands autoclaving at 10 lb. pressure for 10 min., the method of sterilization we normally use; we have no reason to suppose that it would not withstand a longer period of sterilization at a higher temperature. In contrast with the batch variability of bile salts, we have found no corresponding batch variability in Teepol. Colour distinction between lactose and non-lactose fermenters on a mixed plate is less clear on Teepol agar than on MacConkey agar but opacity differences can be taken more into account. For primary isolation of non-lactose fermenting organisms both these media are far inferior to Leifson's medium (1935).

Tables 4 and 5 provide no evidence that Teepol broth is less suitable for counting than MacConkey broth. On the contrary, though all the instances in which the probable counts in MacConkey broth were higher than in Teepol broth can be attributed to sampling error, the converse is not the case. In each of two presumptive coli probable counts on drinking waters, 8 tubes more were positive with Teepol broth. The corresponding mean probable numbers given in the appropriate table of Report (1939) for these waters were as follows: Teepol broth 175 and >1800, MacConkey broth 8 and 50 respectively. The probability of the former 8-tube discrepancy occurring by chance at least once in 166 samples is greater than 0.05, and this discrepancy might therefore have been due to chance alone. The value of  $p$  for the latter discrepancy (which gave probable counts of >1800 and 50 respectively) occurring at least once in 166 samples is however less than 0.0375. It is unlikely that this discrepancy was due to sampling error alone and it is a reasonable assumption that an organism in this water was more viable in Teepol broth than in

MacConkey broth. It is also unlikely that the higher count was attributable to the presence in the water of a Gram-positive lactose-fermenting organism, since Teepol is more inhibitory than sodium taurocholate to Gram-positive organisms.

A detergent for use in culture media in place of bile salts should have the following properties; it should be chemically pure or of constant composition lacking bacteriologically undesirable impurities; it should not be inhibitory to *Salmonella* and *Shigella* species or to pathogenic strains of *Escherichia coli*; it should have relatively high selectivity and anti-swarming factors and it should be easily obtainable and cheap. The ease with which we found a detergent which fulfilled the above criteria suggests that there may well be other detergents equally suitable. It is therefore of interest that lauryl sulphate broth has been widely used in the United States for the presumptive test for coliform bacteria (Pederson & Skinner, 1955).

The last but not least reason why we favour the use of Teepol is that of cost. A 1955 price-list by a well-known manufacturing chemist shows the price of 500 g. of sodium taurocholate (sufficient for 100 l. of medium) as £8. The corresponding cost of sodium tauro-glycocholate is given as £3. 0s. 6d. The cost of Teepol for 100 l. of medium is twopence!

Our thanks are due to Dr P. Armitage of the Medical Research Council's statistical unit for help and advice in the statistical calculations and to Shell Chemicals Ltd., for supplying samples of Teepol and olefine, and for permission to publish details concerning the constitution of Teepol.

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## Microcyst Formation and Germination in *Spirillum lunatum*

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**SUMMARY:** *Spirillum lunatum* has been shown to possess a life cycle in which there is an alternation between a vegetative and a microcyst stage. Microcyst formation may occur from the spiral vegetative cell by: (1) fusion of two entwined organisms to form one or more microcysts; (2) the production of a protuberance at some point along the organism into which the entire organism is gradually absorbed; (3) the gradual shortening and rounding of the organism to form an oval to spherical body. Microcyst formation begins, in a typical broth culture, at c. 24 hr. after inoculation and the majority of the organisms are in the microcyst stage after a 4-day incubation period. When microcysts from an old culture are inoculated into fresh media, they germinate to form the spiral vegetative organism. Germination occurs by either unipolar or bipolar emergence of the germ tube.

During a taxonomic study of the genus *Spirillum* Ehrenberg (Williams, 1952), it was found that three species of the marine spirilla and two of the freshwater species exhibited morphological changes during the growth of the cultures. Young cultures, which consisted entirely of spiral organisms, gradually changed until only oval or spherical bodies (0.8–5.0  $\mu$ . in diameter, depending on the species) were found in old cultures. When these bodies were inoculated into fresh media, the normal spiral organism germinated from them. Since the entire organism is involved in the formation of these oval to spherical bodies, in a manner similar to the formation of the microcysts of the *Sporocytophaga* (Stanier, 1942; Grace, 1951), these bodies have been called 'microcysts'. Because of its relatively large size, *S. lunatum* was chosen as the experimental organism. The morphological changes which occur in this species are similar, although not identical, to those occurring in the other two marine spirilla and in the two freshwater species.

### METHODS

The spirillum was grown in sea-water nutrient broth of the following composition: peptone, 5.0 g.; beef extract, 3.0 g.; yeast autolysate, 3.0 g.; and sea water, 1000 ml. Broth tubes were inoculated with old cultures, ranging in age from 2 to 6 weeks, and incubated at 30°. Organisms removed from the broth cultures at appropriate times between 8 and 72 hr. were used for microcultures. Cultures less than 24 hr. old were centrifuged and the harvested organisms re-suspended in a small amount of sterile sea water. Cultures older than 24 hr.

always form a pellicle and suspensions of the older organisms were prepared by emulsifying a loopful of pellicle in 1 ml. of sterile sea water.

Microcultures were used to obtain photographs of the changes which occurred in the transformation of the spiral organisms into microcysts and to show the germination of the microcysts into the vegetative spiral organisms. They were prepared by placing a drop of suspension on a sterile glass slide and covering the preparation with a sterile O coverslip. Excess fluid was removed with blotting-paper and the preparation sealed with immersion oil. The processes of microcyst formation and germination were observed in individual organisms by dark phase-contrast microscopy and photographed serially as the changes occurred. In the serial photographs the letters 'A', 'B' 'C', . . . , indicate the order of the sequence. The time, in minutes, is shown in the lower right-hand corner with the beginning of a sequence taken as zero time.

## RESULTS

### *Microcyst formation*

*From entwined and fused organisms.* When an old culture of *Spirillum lunatum* is inoculated into fresh medium, there is little evidence of growth until the culture is approximately 24 hr. old. At this time, the culture is found to consist entirely of the spiral organisms considered normal for the genus *Spirillum* (Pl. 1, fig. 1). These spiral organisms divide by transverse fission during the next 10–12 hr. During this time the majority of the organisms are actively motile; however, a few become motionless. When a freely motile organism comes in the vicinity of a motionless one, the motile one appears to become attracted to, and may become entwined about the motionless organism. During the entwining process, which may last for several hours, the organisms are in violent motion and it is not possible to obtain still photographs of the early stages. Eventually the two entwined organisms become motionless, and when this occurs, it can be seen that the two organisms have fused (Pl. 1, fig. 2; Pl. 2, fig. 3). As time elapses, the entwined organisms become shorter and thicker and a swelling occurs (Pl. 1, fig. 2E) or a protuberance forms (Pl. 2, fig. 3C) at the point of fusion, which gradually enlarges, absorbing the rest of the fused organisms, to form the microcysts (Pl. 1, fig. 2; Pl. 2, fig. 3).

In some instances there is only one point of fusion between the two entwined organisms and only a single microcyst is formed, but in the majority of cases more than one microcyst is formed from a pair of fused organisms. The number of microcysts produced appears to be related to the number of fusion points. It is difficult to show both organisms of a fusion pair in their entirety by photographs of a particular focal depth. In the sequence shown in Pl. 2, fig. 3, different points of fusion can be seen in the upper portion of fig. 3A and in the lower portion of fig. 3B. Ultimately, two microcysts were formed from this fusion pair, the upper two in fig. 3I fusing into one.

*From a protuberance.* When a broth culture is approximately 32–36 hr. old, the majority of organisms show an abrupt change in morphology. Transverse fission stops almost entirely and the organisms begin to shorten and become

thicker (Pl. 2, fig. 4; Pl. 3, figs. 8, 9), many of them taking on a crescent shape. The crescent-shaped organism contains a single granule, which is observable in the living organism by both light and phase microscopes. It has been shown that this granule corresponds to the chromatin material of the fixed and stained organism (Williams, 1952). A protuberance may arise from the centre of the crescent-shaped organism which gradually enlarges and absorbs the remainder of the organism (Pl. 2, fig. 5).

Protuberances or swellings may also arise before the formation of the crescent-shaped forms and may occur at or near the ends as well as in the centre of the organisms (Pl. 3, fig. 6). Protuberances, produced at both ends of a single organism, may enlarge and merge into a single body (Pl. 3, fig. 6E). A similar fusion of what appear to be almost fully formed microcysts is shown in Pl. 3, fig. 7. Fusion of microcysts also occurred in the contiguous organisms shown in Pl. 2, fig. 5, although, in this instance, the intermediate stages took place so rapidly that they were not photographed.

*By the shortening of the entire organism.* The majority of organisms neither fuse nor produce protuberances or swellings, but merely undergo a very gradual shortening and rounding, ending up as oval or spherical bodies (Pl. 3, figs. 8, 9).

The production of microcysts in any one culture of *Spirillum lunatum* is not synchronous, and freely motile organisms may be observed in cultures up to 3 weeks of age. For about a week longer, non-motile spiral organisms can still be seen, but after this time about the only formed material found in the cultures are the microcysts, which tend to settle to the bottom of the culture tube as a finely granular sediment (Pl. 4, fig. 10).

#### *Germination of microcysts*

When an old culture of *Spirillum lunatum*, completely in the microcyst stage, is transferred to new medium, no apparent change is observable by phase-contrast microscopy in the microcysts for the first few hours. When such material is fixed and stained, however, extensive changes in the chromatin material can be demonstrated. Eventually, at 6–8 hr. after inoculation, a germ tube emerges from the microcyst, which gradually lengthens until the normal spiral organism is produced. The time interval, from the first observance of the germ tube to the emergence of the complete spiral organism, varies from 3–6 hr. in microculture, but the germination time is considerably shorter in the normal broth culture. In the latter, the majority of microcysts have germinated by 16 hr.

Germination of the microcysts occurs by two methods; unipolar emergence of the germ tube (Pl. 4, fig. 11) and bipolar emergence (Pl. 4, fig. 12). The microcyst portion of the emerging vegetative organism is gradually absorbed and no coat is shed as is the case with some of the spore-forming bacilli and the *Sporocytophaga*.

#### DISCUSSION

The microcysts observed in *Spirillum lunatum* and other species of spirilla are apparently the same as the 'coccoid' bodies reported by earlier investigators (e.g. Ellis, 1903; Dimitroff, 1926), and similar bodies can be seen in



photographs recently published by Cayton & Preston (1955), who describe a new species of spirillum, *S. mancupiense*. With the exception of Dimitroff (1926), who compared the 'coccoid' bodies to those reported by Leishman (1918) for the spirochaetes of relapsing fever, either the microcysts were overlooked or else were dismissed as involution forms. However, from the data presented, it can be concluded that the microcysts represent a definite stage in a life cycle common to many spirilla.

The occurrence of life cycles in members of the Eubacteriales has been proposed from time to time (Lohnis, 1921; Hadley, 1937) but, with the exception of the spore-bacillus-spore cycle of the family Bacillaceae, is not generally accepted by bacteriologists. In the last 15 years, however, studies of L-forms by Klieneberger-Nobel (1949, 1951*a, b*), Dienes & Weinberger (1951), Tulasne (1953), and others have shown that various Eubacteriales may reproduce by methods other than transverse fission.

The fusion forms observed in 20–30 hr. cultures of *Spirillum lunatum* resemble those shown by Klieneberger-Nobel for the Morax–Axenfeld bacillus (her figures 1 to 12; 1951*a*). The protuberances produced by organisms of *S. lunatum* are similar to those shown by Stempen (1955) for *Proteus vulgaris* (his figures 2 and 3). Fusion of organisms has also been shown by Hutchinson & Stempen (1954), but these authors doubted whether this process is a sexual one. Plasmogamy without caryogamy is common in the fungi and the fusion observed with the spirilla does not necessarily represent a sexual fusion, although evidence from fixed and stained preparations (to be presented later) suggests that the fusion of the organisms is followed by nuclear fusion.

The similarity in appearance, mode of formation, and mode of germination between the microcysts of *Spirillum lunatum* and those of the *Sporocytophaga*, together with the fact that the latter organisms have been described, in the past, as having a spiral form, might lead one to suspect that *S. lunatum* is merely a mis-identified *Sporocytophaga*. The presence of polar flagella and a very active swimming motility rules out this possibility (Williams, 1952). More important, the similarities mentioned above suggest that the eubacteria and the myxobacteria may not be as divergent, phylogenetically, as some authorities believe.

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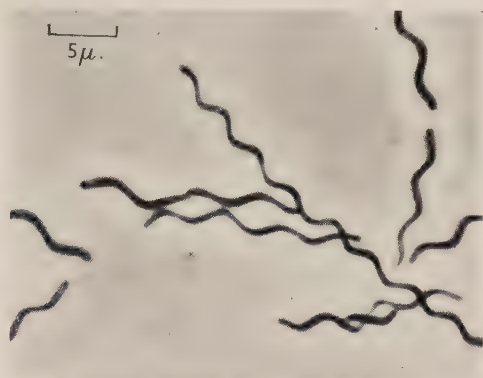


Fig. 1.

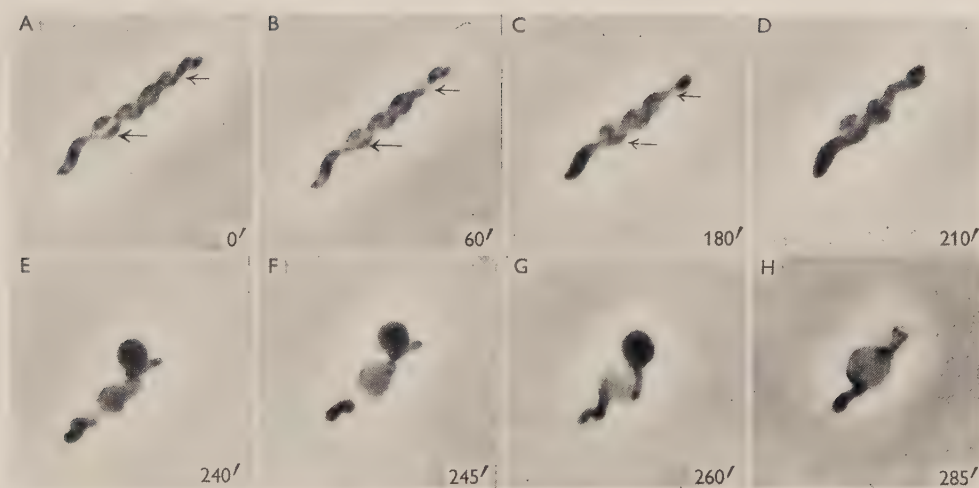


Fig. 2.

M. A. WILLIAMS AND S. C. RITTENBERG—MICROCYSTS IN *SPIRILLUM LUNATUM*. PLATE 1

(Facing p. 208)

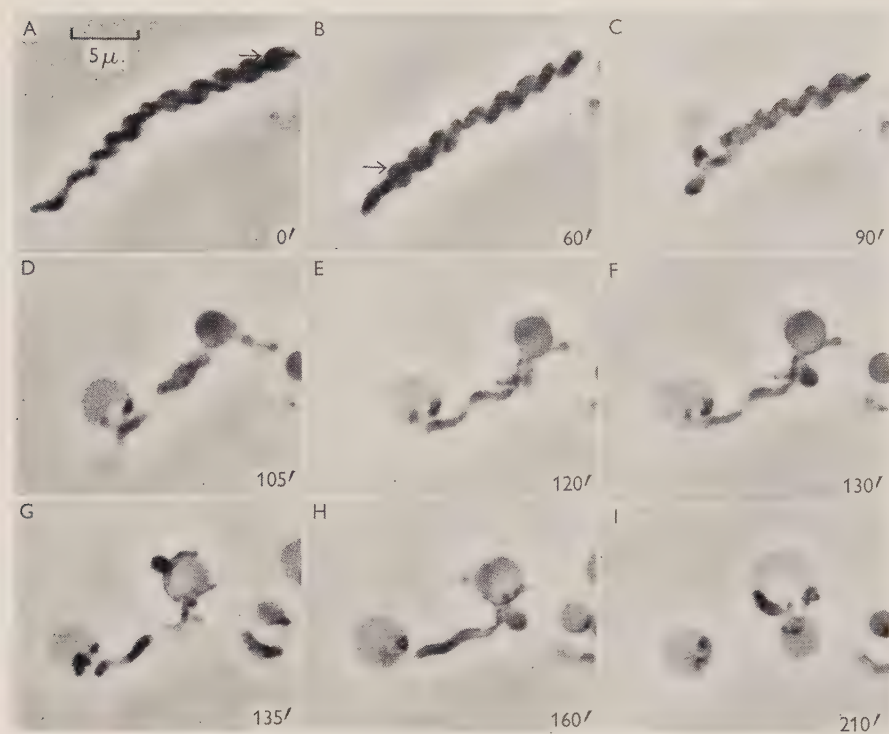


Fig. 3.

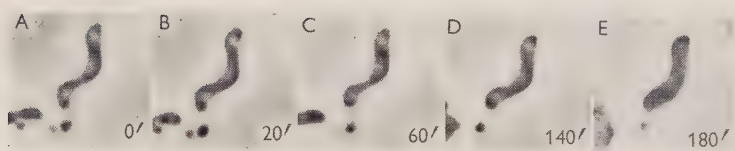


Fig. 4.

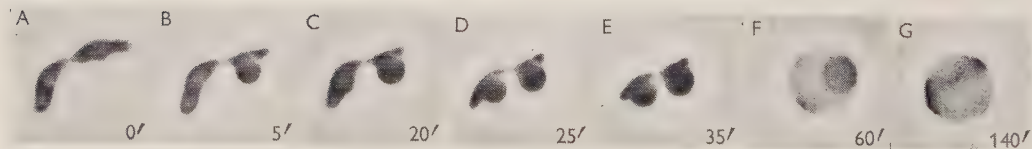


Fig. 5.



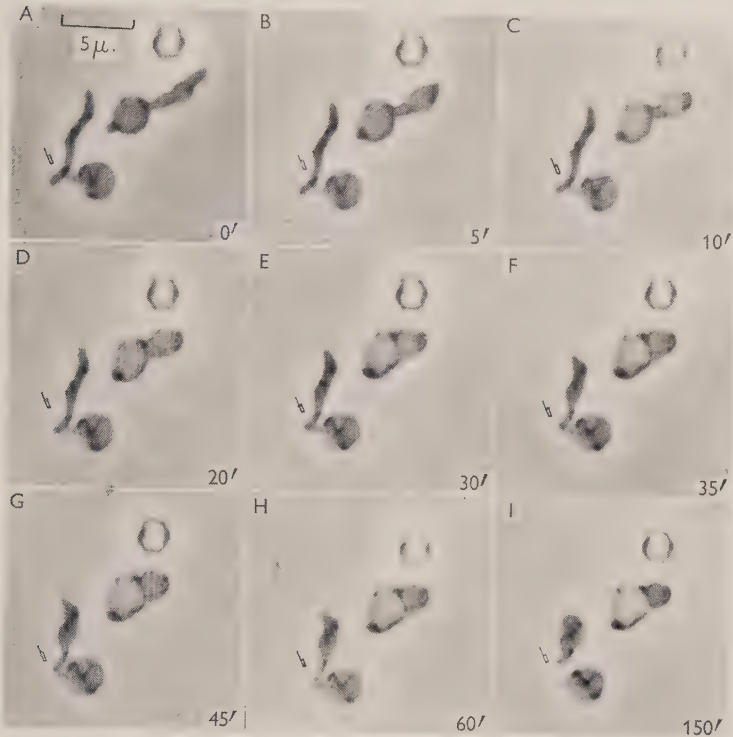


Fig. 6.

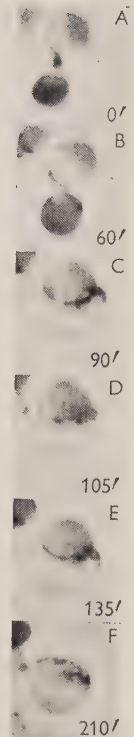


Fig. 7.

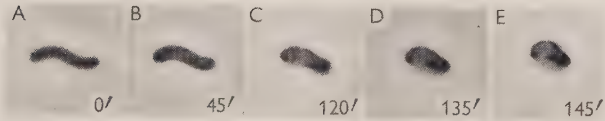


Fig. 8.

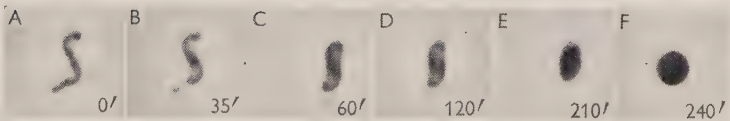


Fig. 9.

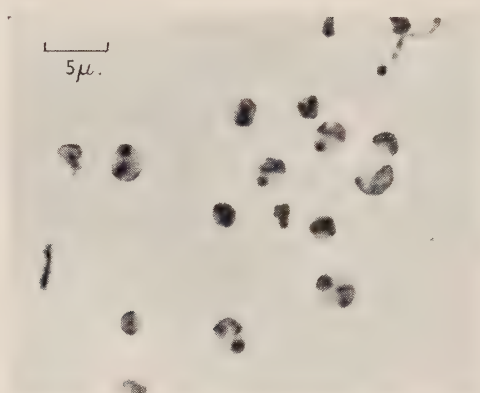


Fig. 10.

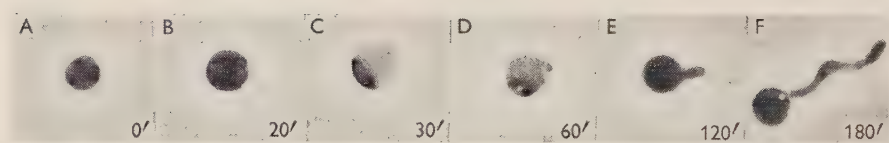


Fig. 11.

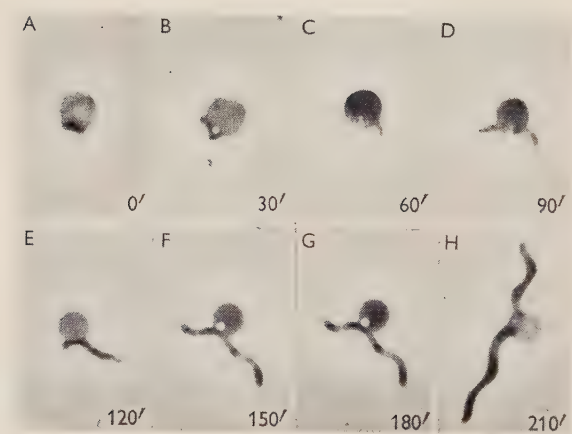


Fig. 12.

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## EXPLANATION OF PLATES

## PLATE 1

All photographs are of living *Spirillum lunatum* made by phase-contrast microscopy. The scale of fig. 1 holds for all photographs.

Fig. 1. Normal vegetative organisms of *S. lunatum*. 26 hr. broth culture.

Fig. 2. Microcyst formation following fusion of organisms. The arrows show the points of fusion. Organisms from a 24 hr. broth culture.

## PLATE 2

Fig. 3. Microcyst formation following fusion. Arrows show upper and lower points of fusion. Organisms from a 26 hr. broth culture.

Fig. 4. Shortening of organisms with production of crescent-shaped form prior to microcyst formation. Organisms from a 30 hr. broth culture.

Fig. 5. Microcyst formation from a protuberance from the middle of a crescent-shaped form. The microcysts from the contiguous organisms have fused. Organisms from a 30 hr. broth culture.

## PLATE 3

Fig. 6. Fusion of microcysts formed from protuberances at opposite ends of organism. Organism 'b' also shows protuberance formation and gradual shortening into a microcyst. Organisms from a 36 hr. broth culture.

Fig. 7. Fusion of two spherical bodies into a single microcyst. Organisms from a 24 hr. broth culture.

Fig. 8. Microcyst formation by shortening and rounding. Organism from a 36 hr. broth culture.

Fig. 9. Microcyst formation by shortening and rounding. Organism from a 24 hr. broth culture.

## PLATE 4

Fig. 10. Microcysts from a 4-week old culture.

Fig. 11. Unipolar germination of microcyst. Microcysts transferred from a 4-week old culture into fresh medium; sequence started after 10 hr. in new culture.

Fig. 12. Bipolar germination of microcyst. Microcysts transferred from a 4-week old culture into fresh medium; sequence started after 12 hr. in new culture.

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## The Infectivity of Extracts made from Leaves at Intervals after Inoculation with Viruses

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**SUMMARY:** When leaves are macerated at intervals after being inoculated with plant viruses, the infectivity of the extracts obtained decreases with increasing time until newly produced virus becomes detectable. Infectivity does not start to increase until approximately twice the time apparently needed for virus to multiply in the epidermis and spread from there to the mesophyll. Epidermal cells infected by inoculation seem to produce too few virus particles to be detected by infectivity tests or else the first-formed particles are unstable *in vitro*.

No evidence was obtained, with Rothamsted tobacco necrosis virus (RTNV) in leaves of tobacco and French bean, that the initial decrease in infectivity occurs because of changes in virus particles that succeed in infecting and causing lesions. If such changes occur they are obscured by the inactivation of particles that do not multiply and cause lesions. Washing inoculated leaves removes 95 % of the inoculated virus, but only slightly decreases the numbers of infections, and adding 'Celite' to the inoculum greatly increases the numbers of lesions without increasing the amount of virus retained by washed leaves. Neither washing nor adding 'Celite' to the inoculum affects the rate at which the infectivity of successive extracts from inoculated leaves decreases. Infectivity continues to decrease after virus appears to have multiplied in and spread from the epidermis.

Cells of *Nicotiana glutinosa* that are infected by tobacco mosaic virus spreading from inoculated epidermal cells die only a few hours after the infectivity of leaf extracts starts to increase: few cells seem to become infected from virus produced in these secondarily infected cells and, at 20°, infectivity reaches a maximum in 2 days. Mesophyll cells of French bean leaves at 22° seem to synthesize new RTNV particles within 5 hr. of becoming infected from the epidermis and to continue synthesizing for another 30 hr., when they probably contain about 10<sup>6</sup> virus particles/cell. Although the cells then die, the virus spreads to further cells and the infectivity of leaf extracts increases for at least five days.

Although Holmes first investigated the infectivity of extracts of tobacco leaves made at intervals after inoculation with tobacco mosaic virus in 1930, only recently have any detailed results been obtained for the hours immediately following inoculation (Bawden & Pirie, 1953; Yarwood, 1952; White, 1954). These results showed that the infectivity of successive extracts of tobacco or *Datura stramonium* leaves usually decreased for some hours after inoculation with tobacco mosaic virus. By contrast, when assays were made on extracts by counting the number of virus particles visible in the electron microscope, no such decrease was detected (Steere, 1952). The experiments described in this paper were made to see whether the initial decrease in infectivity occurs at all generally, and to compare the time needed for infectivity of leaf extracts to increase with that found for other changes which have been detected in inoculated leaves (Bawden & Harrison, 1955).

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## METHODS

The viruses used were Rothamsted tobacco necrosis virus (RTNV), and two strains of tobacco mosaic, the type strain and tomato aucuba mosaic virus. Inoculum of RTNV was infective sap which had been stored frozen; purified preparations of tobacco mosaic and tomato aucuba mosaic viruses were used. The viruses were diluted in distilled water. When 'Celite' (Johns-Manville) was used to increase the number of local lesions, it was mixed in the inoculum. The two primary leaves of French bean plants (*Phaseolus vulgaris* L. var. Prince) were used when almost fully expanded, and the apical shoot was removed. Tobacco plants (*Nicotiana tabacum* L. var. White Burley) were used when they had three leaves of 12–25 cm. in length and *N. glutinosa* L. plants with six leaves of 6–12 cm. in length. The plants were grown in a glasshouse with a mean temperature of 20°.

Inoculations were made by rubbing the upper surface of leaves with the forefinger wet with inoculum. Leaf extracts, made from disks punched out of the leaf blades (Harrison, 1956), were stored frozen, and were thawed and clarified by centrifuging at 10000 r.p.m. for infectivity assays. Infectivity was assayed by comparing the number of local lesions produced by different inocula on a batch of test plants. The treatments were distributed so that each occurred equally on right- and left-hand halves of leaves and at each leaf position. At least six half-leaves were used for each treatment.

Ultraviolet radiation was provided by a low-pressure mercury-discharge lamp (Thermal Syndicate Ltd.); most of the radiation was of 2537 Å. and at 20 cm. from the lamp, the distance used, the radiation intensity was 870  $\mu$ W./cm.<sup>2</sup>.

## RESULTS

*The first detectable virus increase*

To find the shortest time in which newly formed virus became detectable in French bean leaves inoculated with RTNV, infective sap diluted 1/2 and containing 'Celite' was used as the inoculum: the leaves were thoroughly washed after inoculation. About 600 lesions/half-leaf were produced, the maximum number possible. The temperature was about 22°, the optimum for accumulation of RTNV in French bean leaves (Harrison, 1956). Preliminary experiments showed that the infectivity of extracts from such leaves decreased for 6 hr.; in the experiments whose results are given in Table 1, the leaves were not sampled until 3 hr. after they were inoculated. The infectivity of extracts did not increase until between 9 and 10·5 hr. after inoculation; approximately twice the time that the virus seems to need to penetrate into the mesophyll and to be protected from inactivation by ultraviolet radiation (Bawden & Harrison, 1955). If, as seems probable, the time to reach the mesophyll is occupied by virus from the inoculum multiplying in the epidermis, the first lot of virus produced in the epidermis is not detected in leaf extracts, possibly because there is too little of it. The first increase in infectivity may represent the beginning of virus formation in these secondarily infected cells, of which

there will probably be many more than the epidermal cells infected by inoculation. However, another possibility is that the virus as first formed spreads to new cells in the leaf but is unstable *in vitro*, and that forms which are stable in extracts do not occur until later. At 22°, lesions appear in about 40 hr. after inoculation, and the mesophyll cells which die then can only have been infected for 35 hr. at the most. Assuming 5 hr. also is needed for the first virus to be formed in these cells, there is a period of 30 hr. during which RTNV accumulates inside them.

Table 1. *The first detectable increase of tobacco necrosis virus in French bean leaves*

Inoculum was infective French bean sap diluted 1/2 and containing 'Celite'. Total lesions produced on sixteen half-leaves of French bean by sample diluted 1/25 and containing 'Celite' were counted.

Experiment	Time after inoculation (hr.)						Average temp. (° C.)
	3	6	7.5	9	10.5	12	
	Total lesions produced						
A	378	264	356	382	535	840	25
B	329	214	218	295	539	1461	21
C	435	309	329	267	634	814	23
	1142	787	903	944	1708	3115	

Table 2. *The virus content of leaves and effect of irradiation at different times after inoculation*

Host plant: *Nicotiana glutinosa*. Inocula: 100 mg. virus/l.; no 'Celite'. Total lesions produced on twenty-four half-leaves of *Nicotiana glutinosa* by samples diluted 1/5 and containing 'Celite' were counted.

Time after inoculation (hr.)	Tomato aucuba mosaic virus		Tobacco mosaic virus	
	Infectivity of leaf extract (no. lesions)	Lesions developing on irradiated leaves as percentage of control	Infectivity of leaf extract (no. lesions)	Lesions developing on irradiated leaves as percentage of control
1	84	1.5	232	2.6
16.5	60	—	118	—
20	—	50.5	—	61.6
25	60	—	28	—
40.5	59	—	133	—
44	—	97.0*	—	107.0*
49	929	—	1413	—
64	904	—	885	—

\* Lesions visible.

In *Nicotiana glutinosa* leaves inoculated with tobacco mosaic or tomato aucuba mosaic viruses, the time taken for newly produced virus to become detectable in extracts was twice that apparently needed for virus to move from the inoculated epidermis into the mesophyll. Table 2 shows that, with both viruses, the first substantial increase in infectivity occurred between



40.5 and 49 hr. About half the control number of lesions developed on leaves that were irradiated for 3 min. with ultraviolet radiation at 20 hr. after inoculation, and this is taken to be the average time needed for these viruses to multiply in and move from the epidermis into the mesophyll (Bawden & Harrison, 1955). This time is longer than that found by Bawden & Harrison, probably because the experiment was made at a lower temperature; the time taken by RTNV to become insusceptible to ultraviolet radiation in French

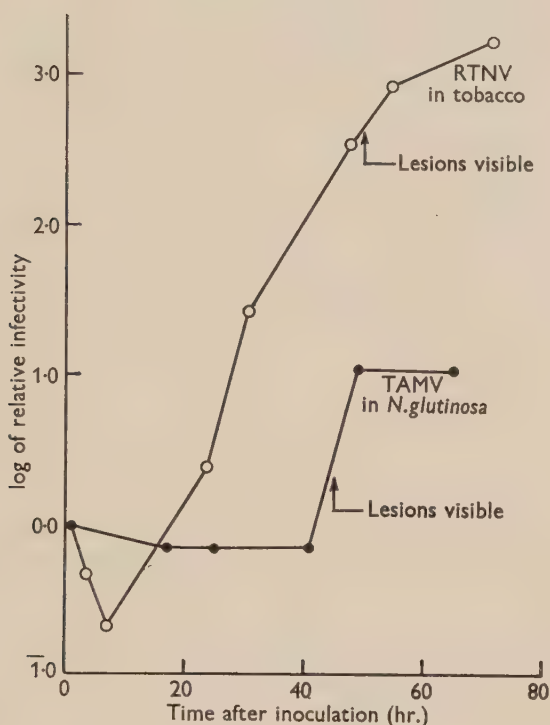


Fig. 1. Increase of tobacco necrosis (RTNV) and tomato aucuba mosaic (TAMV) viruses in inoculated leaves. Leaves washed after inoculation. The inocula were: RTNV, infective tobacco sap diluted 1/4, with 'Celite' added; TAMV, 100 mg./l., no 'Celite'. RTNV was assayed by inoculating French bean, and TAMV in *Nicotiana glutinosa*.

bean leaves varies greatly with change of temperature (Harrison, 1956), and other viruses might be expected to behave similarly. Both tobacco mosaic and tomato aucuba mosaic viruses caused necrotic lesions to appear 45 hr. after inoculation, only a few hours after the infectivity of the leaf extracts had increased detectably. At about 20°, mesophyll cells of *N. glutinosa* leaves seem to synthesize these viruses within 20 hr. of becoming infected and to die when synthesis has gone on for a further 5–10 hr. These processes are accelerated by increasing the temperature, and tomato aucuba mosaic virus multiplies detectably in 21 hr. at 27° and 30° (Harrison, 1956).

Fig. 1 shows that the infectivity of successive extracts from leaves inoculated with RTNV, once it had started to increase, continued to do so for several

days. The necrotic lesions also increased in size during this period. By contrast with tomato aucuba mosaic virus in *Nicotiana glutinosa*, where the infectivity of extracts also decreased at first, infectivity did not increase until 2 days after inoculation; virus multiplication seemed to stop abruptly soon after the lesions appeared. These lesions increased in size subsequently, but only slowly. The apparently constant virus content of the leaves is most simply interpreted as indicating that virus has ceased multiplying, but it may be multiplying with multiplication being balanced by inactivation of virus in dead or dying cells or by the virus becoming less readily extracted from the dead tissue.

About 500 cells are involved in a necrotic lesion caused by tomato aucuba mosaic virus in a leaf of *Nicotiana glutinosa*. Virus seems to multiply in an epidermal cell and then to invade several hundred mesophyll cells, but these are killed and virus produced in them does not repeat the process of invading another large number of cells. When plants with necrotic local lesions are placed at 36°, a temperature at which tomato aucuba mosaic virus produces chlorotic and not necrotic lesions, the virus will invade neighbouring cells (Kassanis, 1952). What restricts the spread of virus in plants at lower temperatures is not known; death of the cells seems an inadequate explanation because when the temperature is raised to 36° virus appears to move from dead to healthy cells.

In tobacco, as in *Nicotiana glutinosa*, when inoculated with tobacco mosaic or tomato aucuba mosaic viruses, the infectivity of successive leaf extracts decreases before it increases. With an inoculum containing 2000 mg. tobacco mosaic virus/l., the relative infectivity of extracts made after 3 hr., 2, 3 and 4 days, was 229, 40, 302 and 865 respectively. Extracts made from tobacco leaves, 2 hr., 1, 2, 3 and 5 days after inoculation with 1000 mg. tomato aucuba mosaic virus/l. produced 22, 6, 7, 13 and 157 lesions respectively when inoculated at a dilution of 1/5 to six half-leaves of tobacco. The times after inoculation when these viruses increased detectably are certainly not the minimum times, because in other experiments tobacco mosaic virus increased between 24 and 31 hr., and Yarwood (1952) detected an increase in 14 hr. at 24°.

#### *Inactivation of the inoculum*

As the three viruses and host plants used all gave qualitatively similar results, it seems to be usual rather than exceptional for the infectivity of extracts from inoculated leaves to decrease before increasing. The phenomenon was therefore studied in some detail with RTNV in tobacco and French bean, to gain information on the factors that affect it and to try to assess its cause and significance. Work with some bacterial viruses shows that they undergo considerable changes while infecting their host cells, and that for approximately half the time between infection and the lysing of the bacteria, these contain no particles that are infective or have the morphology of infective phages. By analogy, then, the loss of infectivity after plant viruses are inoculated to leaves might also reflect some change that virus particles undergo as a preliminary to multiplying in leaf cells. It is equally possible

that virus particles in excess of those that infect and cause lesions may become inactivated on or in the leaf.

Experiments were first made to see how the decrease in infectivity is affected by treatments which increase or decrease the number of lesions produced by a given amount of virus rubbed over the leaves. Tobacco leaves were rubbed, half with infective sap alone and half with sap containing 'Celite'; half of the inoculated leaves in each lot were washed thoroughly 5 min. after inoculation. Newly formed virus was first detected in leaves that were rubbed with sap containing 'Celite', which greatly increases the number of lesions produced. Table 3 shows that 'Celite' did not affect the rate at which the infectivity of extracts decreased, or the proportion of the inoculum that was removed by washing. Washing, which decreases by about 20 % the number of lesions produced, removed about 95 % of the inoculum applied to the leaves. Clearly the inoculum can be considered in two parts. About 5 % is in some way rapidly fixed to the leaf so that it is not removed by washing; this seems to be the only part that is concerned in causing lesions. The proportion of the part that does cause lesions depends on the method of inoculation and is much increased by 'Celite'; it also depends on the physiological state of the leaves, for although the same proportion of the inoculum is retained by washed leaves of various ages and in different physiological states, the numbers of lesions produced differs greatly.

Table 3. *The decrease in residual infectivity of an inoculum applied to tobacco leaves*

Virus: Rothamsted tobacco necrosis. Inoculum was infective French bean sap diluted 1/4. Total lesions produced on eight half-leaves of French bean by samples diluted 1/5 and containing 'Celite' were counted.

Period between inoculation and sampling	Inoculum with 'Celite'		Inoculum without 'Celite'	
	Leaves washed	Not washed	Leaves washed	Not washed
	No. lesions			
3 min.	36	520	34	505
1 hr.	21	314	25	309
5 hr.	27	177	9	223
24 hr.	230	565	17	106

The decrease in infectivity of successive extracts is proportionally the same with washed and unwashed leaves, although extracts from unwashed leaves are more infective. As most of the virus from unwashed leaves is not concerned in producing lesions, the simplest explanation for the phenomenon is that it reflects the loss of infectivity by excess of inoculum. It is unlikely to happen because virus particles become increasingly adsorbed by the leaf surface with the passing of time, for Table 4 shows that as much virus was removed from leaves when washed at 65 min. as when washed 5 min. after inoculation.

Even washed leaves retain far more virus particles than the number of lesions that develops. Indeed, from experiments with inocula containing 'Celite'



and known weights of virus, in my experimental conditions about  $10^5$  particles need to be applied to leaves to get a single lesion. Thus for every one that succeeds in producing a lesion, many fail, and it is the inactivation of the failures that probably causes the decrease in infectivity of successive extracts. Whether the inactivation of virus that cannot be washed from the leaves occurs because of some abortive first step towards multiplication is unknown, but there would seem to be no need to suspect this for the virus that can be removed by washing. For the inactivation of this, there is with RTNV no need even to suppose that the leaf is playing any active part, for much infectivity is also lost when inocula are simply dried in watch-glasses at room temperature and then resuspended in water.

Table 4. *The effect of washing tobacco leaves at intervals after inoculation on the residual infectivity of the inoculum*

Virus: Rothamsted tobacco necrosis. Inoculum was infective French bean sap diluted 1/4 and containing 'Celite'. Total lesions produced on sixteen half-leaves of French bean by samples diluted 1/5 and containing 'Celite' were counted.

Period between inoculation and sampling	Leaves not washed	Washed 5 min. after inoculation	Washed 65 min. after inoculation
	No. lesions		
3 min.	2075	2235	1765
1 hr.	835	67	1310
5 hr.	96	15	15

Tobacco mosaic virus is less affected than RTNV by drying, but if its inactivation in inoculated leaves is a consequence of infection, then the event can occur in apparently immune plants. When French bean leaves were inoculated with tobacco mosaic virus and washed, no lesions developed and no virus increase was detected by infectivity assays on leaf extracts. The infectivity of successive extracts decreased, as with plants in which tobacco mosaic virus multiplies. In one experiment, samples taken 2, 8, 24 and 48 hr. after inoculation produced 280, 127, 78 and 33 lesions respectively when inoculated at a dilution of 1/10 on eight half-leaves of *Nicotiana glutinosa*.

To investigate the effect of light and temperature on the decrease in infectivity, leaves of three batches of plants were inoculated with infective sap diluted 1/25 and extracts of the leaves made 1 and 10 hr. after inoculation. Two batches of plants were kept in the glasshouse, one in daylight and the other in darkness at the same temperature: the third batch was kept in a cool darkroom which was at 14°, 17° and 17·5° in different experiments. Plants kept in the glasshouse for a day in the dark after inoculation produced a third fewer lesions than those kept in the darkroom for a day or in normal glasshouse conditions for the whole period. Darkening leaves after inoculation decreases lesion number (Bawden & Roberts, 1948) and decreasing the temperature increases it (Harrison, 1956); the two effects seem to offset each other. Table 5 shows the results of the infectivity assays on the leaf extracts: in the glasshouse, infectivity decreased by 10 hr. to a fifth of the 1 hr. value, irrespective of

whether the leaves were in light or dark, whereas in the cooler darkroom it only decreased by half. Temperature but not light is important in determining the rate of the decrease in infectivity.

Table 5. *Effect of light and temperature on the decrease in residual infectivity of the inoculum*

Virus: Rothamsted tobacco necrosis. Inoculum was infective French bean sap diluted 1/25. Total lesions produced on forty-eight half-leaves (6 expts.\*) of French bean by samples diluted 1/5 or 1/25 and containing 'Celite' were counted.

Period between inoculation and sampling	Host plant—French bean					
	Glasshouse, light		Glasshouse, dark		Darkroom	
	dil. 1/5	dil. 1/25	dil. 1/5	dil. 1/25	dil. 1/5	dil. 1/25
	No. lesions					
1 hr.	2534	787	2330	711	3833	1190
10 hr.	468	139	584	149	2314	562
% $\left( \frac{10 \text{ hr. figure}}{1 \text{ hr. figure}} \right)$	18.5	17.7	25.1	20.9	60.3	47.2
	18.1 average		23.0 average		53.8 average	

\* Temperatures in the six experiments: A and B—glasshouse 23–27.5° C., average 26; darkroom 14 (constant); C and D—glasshouse 20.5–25.5, average 24; darkroom 17.5; E and F—glasshouse 17–24.5, average 21.5; darkroom 17.

### *The rate of virus increase*

The rate of increase of RTNV in French bean leaves was investigated by sampling the leaves at frequent intervals after inoculation. The results of one such experiment, in which the leaf samples taken contained about 430 lesions or lesion sites and gave extracts of 1 ml. in volume, are shown in Table 6; successive tenfold increases in the infectivity of leaf extracts as measured at 19 hr. after inoculation, occurred in 3, 7.5, 10 and 19 hr. periods, and a further fivefold increase took 30.5 hr. more. The sample taken 89 hr. after inoculation produced 180 lesions on eight half-leaves of test plants at a dilution of 1/15,625, using 'Celite': 0.7 ml. was used in the inoculation. Assuming that  $10^5$  particles are applied for every local lesion produced when 'Celite' is used, the number of

virus particles in the 89 hr. sample was  $\frac{1.0}{0.7} \times 15,625 \times 180 \times 10^5$ . If then it be assumed that 1000 cells were infected at each of the 430 lesions at 89 hr., the average number of virus particles obtained from each cell can be calculated:  $\frac{1.0}{0.7} \times 15,625 \times \frac{10^5 \times 180}{430 \times 1000} = c. 10^6$  particles. It is worth comment that this estimate, for a virus in host cells which it kills, is of the same order as estimates for tobacco mosaic virus in tobacco leaf cells (Harrison, 1955; Nixon, 1956), that is, in cells which continue to live and seem not to be seriously crippled by this quantity of anomalous particles.

Table 6. *Increase of tobacco necrosis virus in French bean leaves*

Inoculum was infective French bean sap diluted 1/15. Total lesions produced on eight half-leaves of French bean were counted.

Time between inoculation and sampling (hr.)	Dilution of sample ('Celite' added)						Relative virus content*
	1/5	1/25	1/125	1/625	1/3125	1/15,625	
1	2+1	—	—	—	—	—	1.5
17	6+5	—	—	—	—	—	5.5
19	5+14	—	—	—	—	—	9.5
22	102	30	—	—	—	—	118
25	569	118	—	—	—	—	550
28	—	172	25	—	—	—	715
41	—	—	563	122	—	—	13,900
45	—	—	772	215	—	—	21,750
49	—	—	—	213	41	—	25,250
65	—	—	—	1474	524	—	284,100
89	—	—	—	—	895	180	501,900

\*  $\frac{a+4.5b}{2} \times \frac{D}{5}$ ;  $a$ =lesion count at lower dilution;  $b$ =lesion count at greater dilution;

$D$ =reciprocal dilution of 'a'.

#### DISCUSSION

The results described in this paper show that when RTNV is multiplying in the epidermal cells of French bean leaves and invading the mesophyll (Bawden & Harrison, 1955) the infectivity of leaf extracts not only does not increase from the time of inoculation but at first actually decreases. Only when newly produced virus starts to accumulate in the mesophyll cells, does there seem to be enough to detect by infectivity assays. Also, the presence of the first newly produced virus is masked by that of the residual inoculum. Some at least of the inoculum virus retained on leaves washed after inoculation seems to be inside wounded cells; Yarwood (1952) found that washing removed 99.9 % of tobacco mosaic virus sprayed on to leaves but only 94 % of that inoculated by rubbing. The residual virus may lose its infectivity and this probably causes the decrease in infectivity of extracts of washed leaves with increasing times after inoculation. Or, instead of virus particles losing infectivity, the number of particles recovered in extracts of the leaves may have decreased. The experiments described above gave no evidence of this and Steere (1952), who counted the numbers of tobacco mosaic virus particles in extracts of tobacco leaves using an electron microscope, found that the numbers remained constant before multiplication was detected. There is no unequivocal evidence that the decrease in infectivity is irrelevant to the process of virus multiplication. It may, however, be caused by virus drying on the leaf and becoming non-infective, or conditions in wounded epidermal cells may favour virus inactivation, possibly of the kind which occurs when French bean leaves infected with RTNV are kept at 30° (Harrison, 1956).

Some of the virus particles in preparations of potato virus X that have been partially inactivated by ultraviolet radiation, multiply when the tobacco test



plants are exposed to daylight after inoculation but not when the plants are kept in darkness (Bawden & Kleczkowski, 1955). Most of the particles reach the light-sensitive state in 30 min. after inoculation and they remain in this state for only 1 hr. more. This suggests that only those particles of potato virus *X* that undergo a change within 1.5 hr. after inoculation multiply and cause lesions. It is dangerous to argue from results obtained with potato virus *X* in tobacco to RTNV in French bean, but the results strongly suggest that changes which occur later than 2-3 hr. after inoculation do not reflect the initial stages of a process that ends in virus multiplication. The fact that RTNV from the inoculum continues to lose infectivity at least until new virus has probably started to accumulate in secondarily infected cells, when ultra-violet irradiation of the leaves prevents few or none of the lesions developing, suggests therefore that most of the decrease in infectivity is separate from the processes which lead to production of new virus.

By analogy with bacteriophage multiplication, it might be expected that soon after infection the virus content of leaves would increase in 'steps', the virus represented by each 'step' coming from a different set of sites of multiplication. This does not happen with RTNV in French bean leaves, probably because the leaf cells continue to produce RTNV for a period several times longer than that needed for production to start. By contrast, the secondarily infected cells of *Nicotiana glutinosa* leaves seem to continue producing tobacco mosaic or tomato aucuba mosaic viruses for only a fraction of the period needed for the first to form, and there is the appearance of a single 'step'. Only one 'step' occurs because the virus formed in the epidermal cells infected at inoculation is not detected, and virus seems to spread to few of the cells which surround the secondarily infected ones; Fig. 1 thus represents the accumulation of virus in secondarily infected cells. Results obtained with tobacco mosaic virus in *Datura stramonium* leaves (White, 1954) can be interpreted similarly.

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## Self-fertility in *Pseudomonas aeruginosa*

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**SUMMARY:** Two interfertile strains of *Pseudomonas aeruginosa* were re-examined for evidence of self-fertility. One strain was self-fertile, the frequency of recombination being affected by the manner in which the parent organisms were grown before being combined. The other strain gave no evidence of self-fertility.

Genetic recombination has been shown to occur in *Pseudomonas aeruginosa* in a manner similar to that which occurs in *Escherichia coli* (Holloway, 1955). One conclusion reached in that work was that the strains used were interfertile in certain combinations but self-sterile. All crosses between mutant strains of the same parental strain were made with organisms grown on agar slopes. Some anomalous results were later obtained with certain inter-strain crosses in which recombination frequencies for bacteria grown in aerated broth were higher than for those grown on agar slopes; this was contrary to earlier experiments. The lack of recombination exhibited in the earlier experiments on intra-strain crosses may therefore have been due to the manner in which the organisms had been grown rather than to an inherent self-sterility. This possibility was strengthened by the findings of Loutit (1955), who obtained results consistent with the occurrence of genetic recombination between biochemical mutants derived from the one parental strain, and in these experiments the bacteria were grown in broth.

### METHODS

Except where stated, the methods and media used were the same as those described previously (Holloway, 1955). Aeration of cultures was achieved by agitation on the shaking machine described by Kantorowicz (1951), at a frequency of 116 oscillations/min., and an amplitude of  $1\frac{1}{4}$  in. Cultures were grown in 100 ml. of Difco nutrient broth + 0.5% Difco yeast extract in 8 oz. medical flat bottles (capacity 235 ml.). The strains used were various auxotrophic mutants of the parent strains I and L described earlier (Holloway, 1955), these parent strains being interfertile when certain auxotrophic mutants are crossed.

As some difficulty had been experienced in maintaining stock cultures in a completely unaltered state, the following procedure was adopted. At the beginning of this set of experiments all the strains to be used were each grown separately in aerated broth overnight, and about 1 ml. quantities of each culture were then sealed in thin-walled glass ampoules. The ampoules were quick-frozen in a solid CO<sub>2</sub> + ethanol mixture and stored at -15°. When a particular strain was required, an ampoule was thawed and used for the inoculum. With this method no variation in stock cultures has yet been detected.



## RESULTS

Crosses were usually set up so that not only could prototrophic recombinants be detected but also recombination for either or both of the non-selective markers streptomycin (S) or chloramphenicol (C). The influence of the manner in which the bacteria were grown before crossing was investigated for a number of crosses. It was found that for most inter-L crosses the frequency of recombination when the bacteria were grown in aerated broth was appreciably higher than when they were grown on agar slopes. The results of a typical experiment are shown in Table 1. Growth in non-aerated broth gave lower recombination values than that for aerated broth, while growing the two strains together in aerated broth conferred no advantage. Although the frequency of recombination when the parents were grown on agar slopes was very low, nevertheless recombination did occur, as was shown by the formation of the non-parental type  $Ad^+T^+S^rC^s$ . This low frequency of recombination when the parents were grown on agar slopes led to the previous erroneous conclusion that strain L was self-sterile.

Table 1. *Variations in recombination frequency and segregation ratios for the markers S and C, for the cross  $L(Ad^-)S^rC^r \times L(T_1^-)S^sC^s$  under different cultural conditions*

Cultural conditions	Recombination frequency*	S and C response among prototrophs			
		$S^rC^r$	$S^sC^r$	$S^rC^s$	$S^sC^s$
Grown together, aerated broth	5.7	13	0	55	4
Grown together, non-aerated broth	1.9	13	0	27	4
Grown separately, aerated broth	16.9	0	0	61	11
Grown separately, non-aerated broth	1.1	7	0	13	7
Grown separately, agar slopes	0.5	6	0	4	1

\* Prototrophs/ $10^9$  parent organisms. Corrected for back mutation to prototrophy of the parents from information derived from plate counts.

A number of crosses were carried out between various mutants of strain L to confirm the presence of genetic recombination in such crosses and to compare the results with the  $1 \times L$  crosses previously examined. These crosses were all made with parents grown overnight in aerated broth, and the results are given in Table 2. There can be no doubt that genetic recombination had occurred, as is seen by the various non-parental types recovered. In the case of the cross  $L(Ad^-) \times L(T_1^-)$ , crosses were set up with the markers S and C entering the cross in all possible combinations. The results indicate that the progeny always tended to resemble the  $L(Ad^-)$  parent with respect to the marker C and the  $L(T_1^-)$  parent with respect to S. In much the same way as was found previously for the  $1 \times L$  crosses, different combinations of selective markers resulted in different recombination frequencies and different ratios of sensitivity and

resistance of S and C. In most cases complete reliance cannot be placed on the observed ratios of S and C amongst the prototrophs tested, for with the low recombination frequencies observed, a certain proportion of the colonies picked off were undoubtedly back-mutations to prototrophy of the parent auxotrophic strains.

Table 2. *Recombination frequencies of L × L matings, and streptomycin and chloramphenicol resistance of the resulting prototrophs*

Cross	Recombination* frequency	S and C response among prototrophs			
		S <sup>r</sup> C <sup>r</sup>	S <sup>s</sup> C <sup>r</sup>	S <sup>r</sup> C <sup>s</sup>	S <sup>s</sup> C <sup>s</sup>
L(IV <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> ) × L(LIL-S <sup>s</sup> C <sup>s</sup> )	3.9	38	1	0	27
L(IV <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> ) × L(Thr-S <sup>s</sup> C <sup>s</sup> )	0	—	—	—	—
L(IV <sub>1</sub> -S <sup>s</sup> C <sup>s</sup> ) × L(T <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> )	2.4	39	204	3	156
L(IV <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> ) × L(Ad-S <sup>s</sup> C <sup>r</sup> )	3.7	2	56	11	2
L(IV <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> ) × L(P-S <sup>s</sup> C <sup>s</sup> )	0.2	—	—	—	—
L(LIL-S <sup>r</sup> C <sup>s</sup> ) × L(P-S <sup>s</sup> C <sup>r</sup> )	0	2	2	58	0
L(LIL-S <sup>s</sup> C <sup>s</sup> ) × L(Ad-S <sup>r</sup> C <sup>r</sup> )	3.8	15	6	5	45
L(LIL-S <sup>r</sup> C <sup>s</sup> ) × L(Thr-S <sup>s</sup> C <sup>r</sup> )	0.1	0	1	71	0
L(Thr-S <sup>s</sup> C <sup>s</sup> ) × L(T <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> )	0.2	—	—	—	—
L(Thr-S <sup>s</sup> C <sup>s</sup> ) × L(Ad-S <sup>r</sup> C <sup>r</sup> )	1.4	3	8	6	11
L(Thr-S <sup>s</sup> C <sup>s</sup> ) × L(P-S <sup>r</sup> C <sup>r</sup> )	0	—	—	—	—
L(T <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> ) × L(Ad-S <sup>s</sup> C <sup>s</sup> )	8.7	11	86	2	7
L(T <sub>1</sub> -S <sup>r</sup> C <sup>s</sup> ) × L(Ad-S <sup>s</sup> C <sup>r</sup> )	25.4	0	1	14	93
L(T <sub>1</sub> -S <sup>s</sup> C <sup>r</sup> ) × L(Ad-S <sup>r</sup> C <sup>s</sup> )	34.3	151	22	7	0
L(T <sub>1</sub> -S <sup>s</sup> C <sup>s</sup> ) × L(Ad-S <sup>r</sup> C <sup>r</sup> )	19.7	2	0	154	24
L(T <sub>1</sub> -S <sup>r</sup> C <sup>r</sup> ) × L(P-S <sup>s</sup> C <sup>s</sup> )	0.7	5	27	1	1
L(Ad-S <sup>r</sup> C <sup>r</sup> ) × L(P-S <sup>s</sup> C <sup>s</sup> )	3.8	0	9	21	42

\* Prototrophs/10<sup>9</sup> parent organisms. Corrected for back mutation to prototrophy of both parents.

With regard to strain 1, crosses were carried out which involved most of the possible matings of the auxotrophic mutants 1(T<sub>1</sub>-), 1(LIL-), 1(IV<sub>1</sub>-), 1(IV<sub>2</sub>-), 1(M<sub>1</sub>-), 1(Se-), 1(M<sub>1</sub>-T<sub>1</sub>-). In no case did the number of prototroph colonies exceed significantly the number of colonies due to back-mutation shown by controls of the parents plated on their own, and in no case was any segregation of the markers S and C demonstrated when the colonies were picked off and tested in the usual way. For strain 1, the previous conclusion of self-sterility is confirmed.

## DISCUSSION

On the basis of the segregation of certain markers in *Pseudomonas aeruginosa* it has previously been found that strain 1 acts as an acceptor strain and strain L as the donor strain. In *Escherichia coli* the donor strain is F<sup>+</sup> and self-fertile while the F<sup>-</sup> acceptor strain is self-sterile. Although it has not been possible to demonstrate any F agent in *Pseudomonas aeruginosa*, the similarity in behaviour between the two mating systems is obvious. Furthermore, it is known that variations in cultural conditions can alter the F status of *E. coli* K12. The effect of cultural conditions on recombination frequency in *P. aeruginosa*

described above is additional evidence for suggesting that the basic mechanism underlying genetic recombination in these two organisms may be similar.

I wish to thank Mrs F. Vagg for her technical assistance.

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## The Statistical Distribution of Phenotypically Modifiable Particles and Host-range Mutants in Populations of Vi-phage II

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**SUMMARY:** The distribution of adapted particles of Vi-phage II has been examined. Fluctuation tests in which phage A was grown for a single cycle in type A of *Salmonella typhi* showed that phage E1 particles conformed to a Poisson distribution in a series of small samples and cannot, therefore, be spontaneous mutants of phage A. Particles of phage D1, on the other hand, showed a clonal distribution in a similar series of samples and are thus spontaneous host-range mutants of phage A. Phage E1 reverted to phage A during a single cycle of growth in type A organisms, which confirms that phage E1 is a phenotypic modification of phage A. In contrast, phage D1 was unaltered by a single cycle of propagation in type A organisms and its mutant nature is thus verified. Phage 29 has also been shown to be a host-range mutant of Vi-phage II. The adsorption of phage A to type E1 is lethal to the bacteria, of which only a small proportion support phage growth. The general applicability of these findings to the numerous adaptations of Vi-phage II is discussed.

It has been shown recently (Anderson & Felix, 1952, 1953*a, b*; Anderson, 1955; Anderson & Fraser, 1955) that the adaptation of Vi-phage II (Craigie & Yen, 1938) to the different Vi-types of *Salmonella typhi* may take any of three forms. First, the adaptation may be a host-induced modification of the type described by Luria & Human (1952), Bertani & Weigle (1953), Weigle & Bertani (1953) and Luria (1953). This change is brought about by the host organisms during phage multiplication, affects every particle formed, and yields phage of which every particle possesses the new host range. When the phage acquires the ability to attack a new host in addition to that on which it was previously propagated, the change can be regarded as adaptive; and the original phage has an approximately constant plating efficiency on the strain on which the new adaptation is to take place. For example, if a phage is already able to attack organism A and is to be adapted to X, its initial efficiency of plating, that is, its titre on X as a fraction of that on A, might be  $10^{-4}$ . If, after adaptation to X, the plating efficiency of the phage on X becomes 1, it can be shown that propagation of the phage on A will reconvert every particle into its original form and its titre on X compared with that on A again becomes  $10^{-4}$ . Such a total change excludes the possibility of the altered host range of the phage being due to the selection of mutants and gave rise to the designation 'host-induced modification' (Luria & Human, 1952) which we have usually referred to as 'phenotypic modification'. This change may be, as Bertani & Weigle (1953) point out, adaptive (phage A  $\xrightarrow{\text{in X organisms}}$  phage AX) or de-adaptive

(phage AX  $\xrightarrow{\text{in A organisms}}$  phage A). In this example identical symbols are used for the phage and host organisms and it is assumed that, whatever changes of host range the phage may undergo, it will always retain in full the ability to attack organism A. The whole cycle of phenotypic change can be summarized thus:

$$\text{phage A} \xrightleftharpoons[\text{in A organisms}]{\text{in X organisms}} \text{phage AX}.$$

It can be seen that this change possesses genetic continuity only when the phage multiplies in the same variety of host organisms. Although it must find a physical representation in the phage deoxyribonucleic acid, the site under host-induced control cannot be regarded as a gene in the accepted sense of the term.

The second type of adaptation which Vi-phage II may undergo expresses itself as a permanent change in host range of the phage. We have referred to this as a 'genotypic' change, and have pointed out in previous publications (Anderson, 1955; Anderson & Fraser, 1955; Anderson, 1956) that there is evidence that the particles of alternative host range pre-exist in concentrated populations of the wild type of Vi-phage II, which is phage A. Using the same type of symbol as before, phage A may have a plating efficiency of  $10^{-4}$  on organism Y. When plaques selected from such a titration are grown with Y cells, phage AY will result, which has an efficiency of plating of 1 on organism Y. When this phage is grown in A cells it is propagated unchanged as phage AY; that is, the change from phage A to phage AY is a mutation affecting the host range of Vi-phage II.

The third type of modification affecting Vi-phage II is a combination of the two forms already described. A host-range mutant may undergo phenotypic modification of the type described earlier.

It has been shown (Anderson, 1955; Anderson & Fraser, 1955) that the genotypic and phenotypic changes of host range in Vi-phage II are independent of each other and that the phenotypic flexibility of the host-range mutants seems to be as great as that of the wild type of Vi-phage II.

The pattern of susceptibility to Vi-phage II in some Vi-types of *Salmonella typhi* has been shown to be partly controlled by symbiotic or temperate phages which are known as type-determining phages (Anderson, 1951, Felix & Anderson, 1951; Anderson & Felix, 1953*b*). Structural formulae for the lysogenically determined Vi-types were suggested by Luria (1953) and this suggestion was developed in recent publications (Anderson, 1955; Anderson & Fraser, 1955). The final proof that a change in the host range of a phage is host-induced or mutational in origin rests on the analysis of single cycles of phage growth in host organisms. Such experiments will be described in the present paper and the statistical distribution of particles showing each type of change will be presented.

#### METHODS

*Media.* 'Difco' broth referred to previously (Anderson & Felix, 1953*a*; Anderson & Fraser, 1955) was used throughout in these experiments; 1.3% New Zealand powdered agar was incorporated in Difco broth for the prepara-

tion of routine agar plates and for the basal layer in agar-layer titrations. The surface layer in agar-layer titrations contained 0.45% of the same agar in Difco broth. One-step growth experiments and phage titrations by the agar-layer technique were carried out by the methods described by Adams (1950).

*Fluctuation tests.* The phage under examination was mixed with a suspension of the host cells in pre-warmed Difco broth in a volume of 10 ml. The suspension consisted of bacteria in the logarithmic phase that had been grown in Difco broth on an agitator. The mixture of phage and bacteria was incubated with agitation at 38.5° for 15 min. (adsorption tube). Two ml. were then removed and centrifuged at 2125 g. for 10 min. and the resulting supernatant fluid was used for the titration of unadsorbed phage. A sample of the remainder of the adsorption tube was then serially diluted so as to yield final suspensions containing the required number of bacteria. This number varied with the type of experiment and will be indicated at relevant points. The final suspension was distributed in amounts of 0.5 or 1 ml. into a number of tubes which were incubated at 38.5° for 120 min. Difco agar, inoculated with bacteria of the Vi-type used as the indicator strain in each experiment, was added to each tube to bring the volume to 3 ml. and the final percentage of agar to 0.45. The tubes were then poured on to separate Difco agar plates and incubated overnight.

## RESULTS

An initial one-step growth experiment (Ellis & Delbrück, 1939; Delbrück, 1940) was carried out to determine the mean burst size of phage A grown on Vi-type A. Fig. 1 shows the results of this experiment. The minimum latent period was 35 min. and the mean burst size about 200 particles. This phage yield is approximately twice that observed by Baron, Formal & Spilman (1955) with the same phage and Vi-type.

### *The range of burst size of phage A in type A cells*

The one-step growth experiment gives an estimate of the mean burst size but provides no information concerning the range over which it may be distributed. Experiments were carried out, therefore, to determine this range. Type A cells infected with phage A in low multiplicity were distributed in a series of 100 tubes so as to give a low probability that any tube contained more than one infected bacterium. The tubes were incubated beyond the latent period and plated on type A to determine the phage yield in each. Table 1 summarizes such an experiment.

30 % of the tubes in the series summarized in Table 1 yielded counts of between 60 and 300 particles. There was a reasonable probability that these tubes contained single phage-infected bacteria and the range of burst size of phage A grown on type A bacteria evidently lies between these limits. Other fluctuation tests, however, have shown that the lower limit may be as few as 30 particles and that the upper may exceed 400. The burst size varies in different host-range mutants; for example, the mean burst size of phage D1 is about half that of phage A.



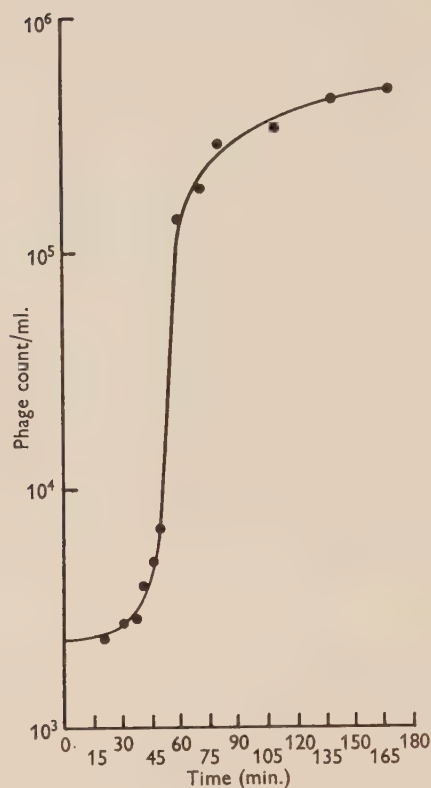


Fig. 1. One-step growth curve of phage A in Vi-type A of *Salmonella typhi*.

Table 1. *Distribution of single bursts of phage A from type A cells*

Adsorption tube; $5.0 \times 10^8$ phage particles + $3.0 \times 10^8$ organisms/ml.	
Phage:organism multiplicity ratio	1.67:1
Free phage after adsorption:	$1.9 \times 10^6$ particles/ml.
Percentage of phage adsorbed:	99.5
Adsorption tube diluted $0.5 \times 10^{-8}$ and 1.0 ml. distributed to each of 100 tubes; all tubes incubated at $38.5^\circ$ for 2 hr.	
Proportion of tubes not yielding bursts of phage	$= 0.54 = e^{-m}$
$m$ = mean number of infected organisms per tube	$= 0.62$
Mean burst size:	212

Theoretical distribution (Poisson) of number of organisms yielding bursts in 100 tubes:

0	(54) (observed)	3	2.1 (calculated)
1	33.4 (calculated)	> 3	0.5 (calculated)
2	10.0 (calculated)		

*The distribution of mutants and phenotypically modifiable particles*

If particles of different host range from the main stock occur as mutants in a phage, they will have a clonal distribution in a series of small samples (Luria & Delbrück, 1943; Luria, 1951). If, on the other hand, they are manifestations of a randomly distributed non-mutational heterogeneity in the phage, or if the phage is qualitatively uniform and the potentiality to adapt any particle to themselves to produce a change in host range is a randomly distributed property of a minority of organisms of the new host, then the plaques of phage

Table 2. *The statistical distribution of particles of phage E1 and phage D1 in a population of phage A grown for a single cycle in type A of Salmonella typhi*

Efficiency of plating of phage A stock on type D1:	$2.1 \times 10^{-4}$
Efficiency of plating of phage A stock on type E1:	$1.1 \times 10^{-3}$
$7 \times 10^8$ phage A particles/ml. } Adsorption tube (AT).	
$5.2 \times 10^7$ type A cells/ml. } Phage: cell multiplicity ratio	13.5:1
AT incubated at $38.5^\circ$ for 15 min. with agitation	
Unadsorbed phage in AT:	$1.25 \times 10^7$ /ml.
Percentage of phage adsorbed:	98.2
Percentage of cells infected (estimated by count of survivors):	99.8
Infective centres on type A before incubation in $10^{-4}$ dilution of AT=first dilution tube (FDT):	$4.4 \times 10^3$ /ml.*
Infective centres on type A before incubation in dilution containing estimated $10^3$ infected cells/ml. (For distribution in 0.5 ml. amounts, incubation and subsequent plating on type D1):	$7.5 \times 10^2$ /ml.*
Infective centres on type A before incubation in dilution containing estimated $2.6 \times 10^2$ infected cells/ml. (For distribution in 0.5 ml. amounts, incubation and subsequent plating on type E1):	$2.5 \times 10^2$ /ml.*
All tubes incubated for 2 hr. at $38.5^\circ$ .	
Mean burst size:	180 particles
Efficiency of plating of phage in FDT after incubation:	
$\frac{\text{titre on type D1}}{\text{titre on type A}}$	$= 1.6 \times 10^{-4}$
$\frac{\text{titre on type E1}}{\text{titre on type A}}$	$= 2.4 \times 10^{-4}$

\* Figures corrected for free phage.

with the new host range will show a Poisson distribution in a series of small samples. It was known from previous work (Anderson & Felix, 1952, 1953*a, c*; Anderson, 1955; Anderson & Fraser, 1955) that certain adaptations of Vi-phage II were purely phenotypic in nature and that these would revert to phage A (which, in routine test dilution,† lyses only type A and appears to represent the wild phenotype of Vi-phage II) when grown on type A. Other adaptations of Vi-phage II were known to be genotypically stable. An examination was carried out of the statistical distribution of particles of

† In the Vi-phage typing method each phage is used in the routine test dilution, which is the highest dilution giving confluent or semi-confluent lysis on the homologous type of *Salmonella typhi*.

each type of host-range modification in a stock of phage A grown for a single cycle in type A organisms. The new host ranges chosen for scrutiny were: E1 (phenotypic) and D1 (host-range mutant). The plating efficiency of phage A on type E1 is about  $10^{-3}$ ; that of phage A on type D1 is about  $2.0 \times 10^{-4}$ . The fluctuation tests were carried out as described earlier. Phage A was adsorbed to type A bacteria and the suspension was suitably

Table 3. *Distribution of plaques after incubation in tubes each containing 0.5 ml.*

From $3.8 \times 10^2$ infected type A bacteria/ sample. Yield of phage Mean total yield/sample plated on type A: $1.0 \times 10^5$ particles. Distribution of plaques in 50 samples plated on type D1		From $1.3 \times 10^2$ infected type A bacteria/ sample. Yield of phage Mean total yield/sample plated on type A: $3.5 \times 10^4$ particles. Distribution of plaques in 50 samples plated on type E1	
No. of plaques	Frequency	No. of plaques	Frequency
0	19	0	4
1	13	1	12
2	2	2	13
3	0	3	11
4	2	4	5
5	4	5	4
6	1	6	1
7	0		
8	1	Mean number of plaques/sample on type E1 = 2.34 (observed)	
14	1	= 2.53 (calculated: Poisson)	
17	1	$\chi^2$	= 44.96
24	1	P	= 0.65
33	1	Poisson distribution.	
35	1	Probability of production of particles	
63	1	plating on type E1 per reduplication of	
95	1	phage A = $7.2 \times 10^{-5}$ .	
122	1		
Mean number of plaques/sample on type D1 = 9.2			
$\chi^2$	= 1210		
P	= $\leq 10^{-9}$		
Clonal distribution			
Mutation rate calculated from the pro- portion of tubes showing no mutants = $0.97 \times 10^{-6}$ .			

diluted and distributed in 0.5 ml. amounts into 120 tubes which were incubated for 2 hr. at  $38.5^\circ$ . Fifty of the samples were then plated *in toto* on type E1, fifty on type D1 and, after dilution, twenty on type A to estimate the total yield of phage/sample. Tables 2 and 3 show the results of this experiment. It can be seen from Table 3 that the distribution of plaques on type D1 was clonal in character, which suggests that the particles forming plaques on this strain were mutants that arose spontaneously in populations of phage A, independently of the presence of type D1 organisms.



In contrast to these results are those obtained with the samples plated on type E1. Here the plaques showed a Poisson distribution and it may be concluded that they represented particles undergoing phenotypic ('host-induced') modification after being plated on type E1.

Thus, it was possible to demonstrate the presence of host-range mutants and of particles undergoing phenotypic adaptation to a new host in the same stock of phage A resulting from a single cycle of growth in type A organisms.

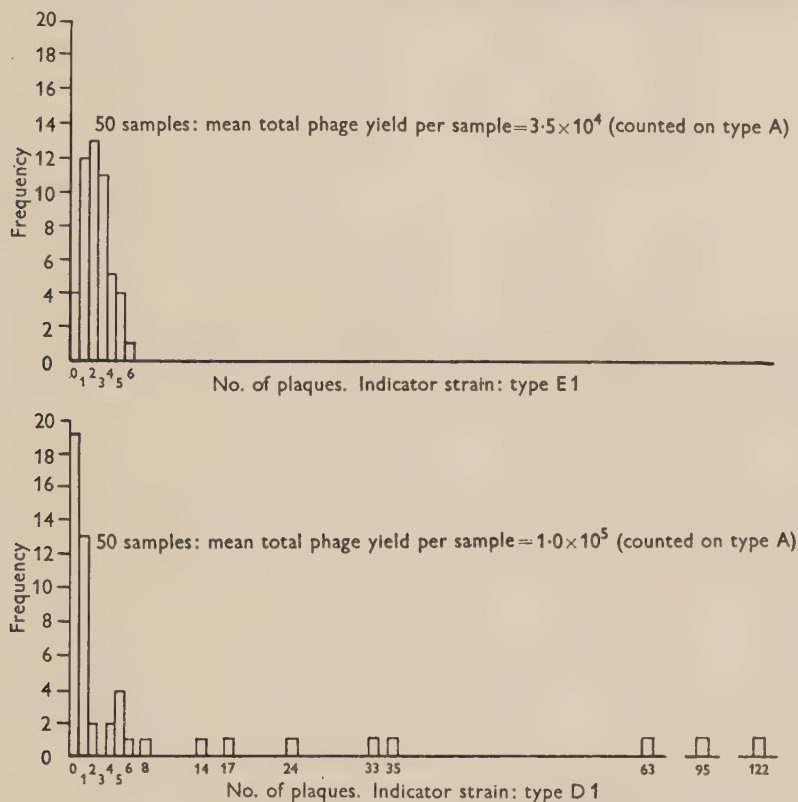


Fig. 2. Fluctuation test (see Tables 2 and 3). Frequency distribution of plaques from single cycle of growth of phage A in type A of *Salmonella typhi*.

Figure 2 is a graphical representation of the distribution of the two types of adapted particle in this experiment. It is known from previously quoted work that both types of change, either separately or in combination, are represented in the Vi-phage typing preparations that have proved so useful in the epidemiology of typhoid fever.

Plate 1 demonstrates effectively the clonal occurrence of the host-range mutant type of particle in phage A stocks. In some of the early experiments the incubation time of the distributed samples was inadequate to ensure that all phage-infected type A bacteria would burst before plating. The period of incubation was later extended to overcome this objection. However, in one of

the insufficiently incubated batches, an infected organism apparently burst as the surface layer of agar was being poured. The tilting and rotation of the plate distributed the phage particles from the origin of the burst and each produced a plaque on the indicator strain, which was type D1. The total number of plaques in this burst was at least 21, and it is evident that the group represented a clone of phage D1 particles produced by a mutation of phage A growing in a type A organism.

From the data in Tables 2 and 3 it is possible to estimate the mutation rate of phage A to phage D1. As the mean total yield of phage/sample in the series plated on type D1 was  $1.0 \times 10^5$  and the proportion of tubes not showing plaques on type D1 organisms was  $19/50 = 0.38$ , the mean number of D1 mutations/tube was 0.97, assuming the frequency of mutation to conform to a Poisson distribution. If, as Luria (1951) suggests, phage multiplies exponentially, the mutation rate to phage D1 per reduplication of phage A in this experiment was  $0.97 \times 10^{-5}$ . There was a probability of a mean contribution of 1 particle of phage D1/sample from the original stock of phage A, but experiments indicated that such particles were unlikely to be able to produce bursts of phage D1 from type A organisms in the presence of the greatly superior numbers of phage A particles (about 13) infecting the same organisms. The fluctuation test was repeated, using a mean multiplicity of 1 phage A particle/type A organism; the results were corrected by subtraction of bursts which might have been produced by infection of the type A cells with phage D1 particles already present in the original stock of phage A. The phage multiplicity ratio in this experiment was sufficiently low to allow pre-existing phage D1 particles to multiply in the bacteria they infected. After the correction had been applied, this fluctuation test yielded a figure similar to that given in Table 3 for the mutation rate of phage A to phage D1.

It was found that when phage A was applied to a lawn of type D1 on agar plates, the host range of a number of single plaques picked at random could be divided into two classes which occurred with approximately equal frequency. Class 1 lysed only type D1, whereas class 2 lysed both types D1 and D5. Table 4 shows the sensitivity of types D1 and D5 to their homologous typing phages and to the two classes of plaques picked from plates on which phage A had been titrated on type D1.

It must be pointed out that phage D5 is normally prepared by adapting phage A to type D5; it was shown in earlier work (Anderson & Felix, 1953*a*) to be a host-range mutant of Vi-phage II. It is clear from Table 4 that class 1 plaques were identical with phage D1, and class 2 with phage D5. Type D1 selects either mutant indifferently because it is as sensitive to phage D5 as it is to phage D1. This is additional proof that the production of the host-range mutants of Vi-phage II is independent of the presence of the Vi-types of *Salmonella typhi* which they can lyse. The ability to lyse type D5, which is always accompanied by the ability to lyse type D1, can be regarded as an unselected marker when the indicator strain is type D1.

It is thus possible that roughly half the mutations of phage A to phage D1 recorded in Table 3 were to phage D5. The true mutation rate to phage D1

would thus be about 50% of the calculated rate. However, no attempt was made to distinguish phage D1 from phage D5 in the experiment recorded in Tables 2 and 3.

Table 4. *Reactions of phages D1 and D5 and of the two classes of plaques picked from adaptations of phage A to type D1 of Salmonella typhi*

Indicator strains of <i>Salmonella typhi</i>	Typing phages		Plaques picked from plating of phage A on type D1	
	D1	D5	Class 1	Class 2
D1	+	+	+	+
D5	-	+	-	+

+ = lysis; - = no lysis.

*Effect on phenotypically changed preparations and host-range mutants of single cycles of growth on type A*

In earlier publications (Anderson & Felix, 1952, 1953*a*, *c*; Anderson, 1955; Anderson & Fraser, 1955; Anderson, 1956) it was shown that the difference between phenotypically-changed particles such as those of phage E1, and genotypically-changed stocks such as phage D1 can be demonstrated by growing each on type A, when the phenotypically-changed stocks revert uniformly to phage A while the host-range mutants, if they have not previously undergone phenotypic modification, are propagated unchanged. These experiments were carried out by titrating the respective phages on type A, and selecting and propagating discrete plaques. The resulting preparations thus represented an unknown number of cycles of phage growth. Single-cycle experiments were carried out in order to demonstrate unequivocally the difference between the two types of adaptation represented by the starting phages. The respective phages were adsorbed to type A organisms and the suspensions diluted so as to give only a few organisms/ml. One ml. amounts were distributed into fifty tubes. After incubation for 2 hr. to ensure that all infected bacteria had lysed, 0.5 ml. of each tube was plated on type A, and 0.5 ml. on the correct indicator strain for the phage under test: type E1 for phage E1 and type D1 for phage D1. In addition, a dilution containing about 100 infected organisms/ml. was incubated in bulk for 2 hr. and titrated on the relevant indicator strains. Tables 5 and 6 show the results of these experiments. Only the findings on the dilutions incubated in bulk are given.

The platings of the multiple small samples, which had a mean content of about five bacteria each, showed that the host range of the phage-yield from individual bacteria agreed exactly with that from the bulk dilution. Thus, it is apparent that the change from phage E1 to phage A, with loss of the ability to multiply in type E1 bacteria, occurred in a single cycle of growth in type A. On the other hand, phage D1 after growth on type A preserved its host range unchanged, retaining an equal efficiency of plating on type A and D1.



The experiments summarized in Tables 5 and 6 indicate the justifiability of the authors' earlier assumptions concerning the phenotypic nature of certain of the Vi-phage II adaptations (see Anderson & Fraser, 1955) and also confirm the stability of host range of those preparations which have been previously

Table 5. *Single cycle of growth of phage E1 in type A of Salmonella typhi*

6.4 × 10 <sup>8</sup> organisms + 4.8 × 10 <sup>8</sup> phage E1 particles/ml. After 15 min. adsorption at 38.5°, the suspension was diluted 10 <sup>-6</sup> and incubated for 2 hr. at 38.5° = Second dilution tube (SDT).	
Phage adsorbed (%):	99.3
Infective centres in SDT, before incubation, on types A and E1:	3.0 × 10 <sup>2</sup> /ml.*
Efficiency of plating of phage E1: $\frac{\text{titre on type E1}}{\text{titre on type A}}$	= 1.0
Infective centres in SDT after incubation (1) On type A (2) On type E1	4.5 × 10 <sup>4</sup> /ml.* 76/ml.
Efficiency of plating of phage E1 after growth on type A $\frac{\text{titre on type E1}}{\text{titre on type A}}$	= 1.7 × 10 <sup>-3</sup>
Mean burst size:	150 particles

\* Figures corrected for free phage.

Table 6. *Single cycle of growth of phage D1 in type A of Salmonella typhi*

6.2 × 10 <sup>8</sup> organisms + 6.7 × 10 <sup>8</sup> phage D1 particles/ml. After 15 min. adsorption at 38.5°, the suspension was diluted 10 <sup>-6</sup> and incubated for 2 hr. at 38.5° = Second dilution tube (SDT)	
Phage adsorbed (%):	96.0
Infective centres in SDT, before incubation, on types A and D1:	4.0 × 10 <sup>2</sup> /ml.*
Efficiency of plating of phage D1: $\frac{\text{titre on type D1}}{\text{titre on type A}}$	= 1.0
Infective centres in SDT after incubation (1) On type A: (2) On type D1:	4.3 × 10 <sup>4</sup> /ml.* 4.4 × 10 <sup>4</sup> /ml.
Efficiency of plating of phage D1 after growth on type A $\frac{\text{titre on type D1}}{\text{titre on type A}}$	= 1.0
Mean burst size:	107 particles

\* Figures corrected for free phage.

regarded as mutants. It is, of course, known that both changes may be represented in a single preparation of Vi-phage II, and such a phage has a wider host range than those in which purely phenotypic-on-wild-type or purely genotypic changes have occurred, because the host ranges covered by the phenotypic and genotypic changes are summated in each particle. A full discussion of this subject was given by the present authors in a recent paper (Anderson & Fraser, 1955).

*Effect of adsorption of Vi-phage II preparations on heterologous Vi-types*

Although the adsorption of a phage to an organism to which it can become phenotypically adapted is usually as efficient before adaptation as after, the effect of such adsorption on the host varies with the different phage host systems. For example, Bertani & Weigle (1953) found that phage P2, isolated from the lysogenic *Escherichia coli* of Lisbonne and Carrère (Bertani, 1951), when grown on the indicator strain of *Shigella shigae* (Sh), was unable to lyse *E. coli* B. Nevertheless, P2Sh, that is, phage P2 adapted to strain Sh, was readily adsorbed by *E. coli* B but did not affect its growth. On the other hand, Luria & Human (1952) and Luria (1953) found that the phenotypic modification of phage T2 known as T\*2, which was adsorbed to *E. coli* B or B/40 (a mutant of *E. coli* B) without causing lysis or phage reproduction, killed the organisms to which it became attached.

Experiments were carried out to determine the effect of the adsorption of phage A on type E1 of *Salmonella typhi* using phage:host cell multiplicity ratios of 150:1, 15:1 and 1.5:1. The concentration of type E1 bacteria in the adsorption mixtures was  $1.7 \times 10^8$ . Adsorption was carried out for 15 min. at  $38.5^\circ$  with agitation. The concentration of unadsorbed phage was then estimated by centrifuging 2 ml. of the suspension at 2125 g for 10 min. and titrating the supernatant on type A. The number of surviving bacteria was determined by the method of Miles & Misra (1938). The total number of infective centres, that is, free phage + infected bacteria yielding phage, was calculated by plate counts on types A and E1. Dilutions were made in chilled broth and all manipulations were completed during the latent period, so that infective centres registering on type E1 represented bacteria of type E1 infected in the adsorption mixtures at the commencement of the experiment. The infected organisms which produced plaques on type E1 would obviously have yielded bursts of phage E1 if they had been allowed to lyse before plating. The actual phage A concentrations used were  $2.56 \times 10^{10}$ ,  $2.56 \times 10^9$  and  $2.56 \times 10^8$ /ml. The average efficiency of plating of phage A on type E1 is about  $10^{-3}$ . Theoretically, therefore, the maximum number of particles in the phage A stocks in the three successive experiments which could function as phage E1 particles was of the order of  $2.3 \times 10^7$ ,  $2.3 \times 10^6$  and  $2.3 \times 10^5$ /ml. respectively. As the starting concentration of bacteria was  $1.7 \times 10^8$ , not more than 14% of type E1 organisms could have been killed by particles of phage A capable of multiplying as phage E1 in the highest phage concentration used. Thus, any appreciable killing effect on type E1 organisms would be due to adsorption of phage A without subsequent phage multiplication. The results of these experiments are summarized in Table 7. The estimation of the fraction of infected type E1 bacteria functioning as infective centres was based entirely on the plating results on type E1 organisms. It is apparent from Table 7 that adsorption of phage A was lethal to type E1, although multiplication of the phage occurred in only a minority of organisms.

Table 7. *Lethal effect of phage A on Vi-type E1 of Salmonella typhi*

Initial concentration of type E1 organisms:  $1.7 \times 10^8$ /ml. Multiplicities of phage A adsorbed to the bacteria: Expt. 1, 150:1; Expt. 2, 15:1; Expt. 3, 1.5:1. Adsorption: 15 min. at  $38.5^\circ$ .

	Expt. 1	Expt. 2	Expt. 3
Initial phage concentration	$2.56 \times 10^{10}$ /ml.	$2.56 \times 10^9$ /ml.	$2.56 \times 10^8$ /ml.
Free phage after adsorption	$3.6 \times 10^8$ /ml.	$6.2 \times 10^6$ /ml.	$4.1 \times 10^5$ /ml.
Phage adsorbed (%)	98.6	99.8	99.8
Surviving bacteria	$5.3 \times 10^4$ /ml.	$3.6 \times 10^7$ /ml.	$1.3 \times 10^8$ /ml.
Bacteria killed by phage infection (%)	> 99.9	79 ( $= 1.34 \times 10^8$ /ml.)	24 ( $= 4.0 \times 10^7$ /ml.)
Infective centres on type E1	$1.25 \times 10^6$ /ml.	$5.4 \times 10^4$ /ml.	$3.67 \times 10^4$ /ml.
Killed bacteria not yielding phage (%)	99.3	> 99.9	99.9

## DISCUSSION

When Craigie & Yen (1938) first described the Vi-phage typing method they suggested that the adapted preparations of Vi-phage II originated from host-range mutants of the phage that were selected by the specific types of *Salmonella typhi* on which they could grow. This explanation was rejected by Felix (1949) on the grounds that it was unlikely that two sets of complementary mutants could have been evolved in such widely different organisms as the typhoid bacillus and Vi-phage II. The experiments described in this paper show that the adaptation of Vi-phage II to the various Vi-types of *Salmonella typhi* consists of the two different processes of host-induced modification and selection of host-range mutants of the phage, which may occur separately or in combination. Phage D1 was chosen at random for scrutiny as a host-range mutant. An examination of the mutation of phage A to phage 29 has yielded similar results. It is reasonable to conclude, therefore, that all the specific adapted preparations which differ in genotype from phage A are host-range mutants of Vi-phage II. Thus, Craigie & Yen's original hypothesis is partly correct. The demonstration that any preparation of Vi-phage II, either mutant or wild-type, is capable of the same phenotypic plasticity (Anderson & Fraser, 1955) shows that the phage is also able to extend its host range widely without mutational changes. However, the suggestion put forward by Anderson & Felix (1953*a, c*) that the entire range of Vi-phage II adaptations are modifications of the phage phenotype only is incorrect.

There are at least 21 Vi-types (including two that have recently been discovered) that can be lysed only by host-range mutants of phage A. In 13 of these the Vi-type specificity has been shown to depend partly on lysogenicity (Anderson, 1951, Felix & Anderson, 1951, Anderson & Felix, 1953*b*) and the types concerned can be manufactured artificially by lysogenizing suitable non-lysogenic types\* with the correct temperate type-determining phages.

\* The term 'non-lysogenic' is used only in relation to the absence of type-determining phages. Temperate phages that do not have a type-determining function are commonly found in *Salmonella typhi*; these are not considered here.



Thus, these determining phages evidently offer an obstacle to the multiplication of the wild genotype of Vi-phage II which can only be overcome by host-range mutants of the Vi-phage. The identity of each type-determining phage decides which mutant of Vi-phage II shall be selected. In some cases the identification of the particular mutant of Vi-phage II selected offers the only known distinguishing feature between determining phages. For example, when type A of *Salmonella typhi* carries phage f2 it becomes type 29 (structural formula A(f2)). This type is sensitive to, among others, phages 29 and D6. When type A is lysogenized with phage d6, however, it becomes type D6, which is structurally A(d6) and this complex is sensitive to phage D6 but relatively resistant to phage 29. Phages 29 and D6 are different host-range mutants of Vi-phage II and which of the two is selected depends on whether the selecting host is carrying phage f2 or phage d6. As type 29 is equally sensitive to phages 29 and D6, it is evident that if these mutants occurred at equal rates in phage A, they would be selected indiscriminately when phage A was applied to type 29. However, phage A mutates to phage 29 with considerably higher frequency than it does to phage D6. This gives phage 29 a selective advantage over phage D6 when concentrated stocks of phage A are applied to type 29. Phages f2 and d6 are serologically indistinguishable, show identical properties of lability to heat and chemical agents, have identical host ranges and protect the strains they lysogenize against each other. They are distinguishable only by the identification of the host ranges of the mutants of Vi-phage II selected by the bacteria they have lysogenized.

It has been pointed out (Anderson, 1955; Anderson & Fraser, 1955) that the lysogenically-determined types can be regarded as consisting of two components: the non-lysogenic precursor of the type; and the type-determining phage that is presumably associated with the bacterial nucleus. The former is responsible for the phenotypic modification of Vi-phage II; the latter blocks the multiplication of the wild genotype of the Vi-phage and determines which host-range mutant of it shall be selected. These two functions are independent of one another and each must be represented physically in both the organism and the Vi-phage. It can thus be postulated that there are two steps in the multiplication of Vi-phage II that are capable of undergoing changes of a definable nature. One, which is responsible for the changes of the phage in phenotype only, is controlled by a complementary site in the host organism that is unaffected by lysogenization with the type-determining phages. The effect of this site on Vi-phage II can be regarded as a permanent characteristic of the host organism. The effect on Vi-phage II of the site in the bacterium controlled by the type-determining phage depends on the particular determining phage concerned. Each determining phage erects a specific barrier to the wild genotype of Vi-phage II that can only be overcome by a particular host-range mutant or by closely related mutants.

All Vi-types of *Salmonella typhi* are able to adsorb all adaptations of Vi-phage II irrespective of their specificity. The experiments with the adsorption of phage A to type E1 described in this paper show that such adsorption is only

followed by phage multiplication when the phage is of suitable phenotype and genotype. This indicates that the steps in Vi-phage II multiplication that are governed by the phenotypic and genotypic characters examined are distinct from, and follow, adsorption of the phage to the host organism.

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E. S. ANDERSON AND A. FRASER—THE DISTRIBUTION OF ADAPTED PARTICLES OF VI-PHAGE II. PLATE I

(Facing p. 239)

- LURIA, S. E. & HUMAN, M. L. (1952). A non-hereditary, host-induced variation of bacterial viruses. *J. Bact.* **64**, 557.
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EXPLANATION OF PLATE

Clone of phage D1 released during single cycle of growth of phage A in type A of *Salmonella typhi*. Total number of phage particles in sample after burst: ca.  $10^5$ . Indicator strain type D1 *Salmonella typhi*. Magnification,  $\times 10$ .

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SREENIVASAN, A. & VENKATARAMAN, R. (1956). *J. gen. Microbiol.* **15**, 241-247

## Marine Denitrifying Bacteria from South India

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**SUMMARY:** Thirty-two isolates from a number obtained from sea water off the South Indian coast, from marine sand and from molluscs, proved to be denitrifiers. The reactions of 20 of these strains on different media show that they fall into five groups; all are considered to be species of *Pseudomonas*, and two of the groups to be new species. It is possible that the observed fluctuations in the nitrate content of sea water are in part due to the activities of denitrifying bacteria.

The true denitrifying bacteria, which reduce nitrates to nitrous oxide and nitrogen, alone cause losses of nitrogen in the nitrogen cycle. The waters of tropical seas appear to be deficient in nitrates (Harvey, 1945; Jayaraman, 1954). Jayaraman believed that the 'seasonal variations in the nitrate content may be due partly to the fluctuations in the number of denitrifiers'. It is possible that tropical waters are poor in nitrates because of denitrification. In this paper, some denitrifying bacteria from South Indian seas are described.

### METHODS

The denitrifiers were isolated by plating, usually on sea-water agar, and picking off colonies. The details are given in Table 2. Media for testing denitrifying power were all made up in sea water (see Table 1), as were the media used for classification tests (see Appendix). Aerobic cultures on nitrate and nitrite media were grown in test tubes containing Durham tubes, usually at 37°. For anaerobic cultures, the medium was boiled and cooled before inoculation, and was covered with sterile paraffin.

Nitrite was determined by the Griess-Ilosvay test.

Gas was detected by Durham tubes.

### RESULTS

*Occurrence of denitrifiers.* Denitrifying bacteria could always be isolated from 1 ml. samples of sea water, collected 10 miles off Tuticorin (Madras State), and also collected offshore from Calicut in the Arabian Sea. Of 95 isolates from this locality, 13 were denitrifiers, 9 of which were strictly 'marine' forms (ZoBell, 1948); 2 out of 19 isolates from sea water off Tuticorin were also denitrifiers. The dissolved oxygen content of sea water from which the denitrifiers were isolated was in the range 4.0-6.3 mg./l., and the temperature was 27.1-30.0°. Marine sand collected off Tuticorin contained denitrifiers (3 out of 21 isolates), which could be obtained from 1 g. samples. Denitrifiers were also isolated from green mussels (*Mytilus edulis*), collected inshore off Calicut, and from 1/1000 dilutions made from benthic mollusca such as Chanks (*Turbinella pyrum*) and

Pearl Oysters (*Pinctada vulgaris*) collected off Tuticorin. Some more denitrifiers were isolated on a chitin medium, and three copper-tolerant strains from marine molluscs were also found to be denitrifiers (Sreenivasan, 1956).

Table 1. *Composition of media used*

All media made up in aged autoclaved sea water

(1) Broth media				
Ingredient (g./l.)	1 % nitrate broth	0.2 % nitrate broth	0.2 % nitrite broth	0.5 % nitrite- 0.2 % nitrate broth
KNO <sub>3</sub>	10	2	0	2
NaNO <sub>2</sub>	0	0	2	5
Meat extract	3	3	3	3
Peptone	5	5	5	5
NaCl	0	0	0	0
pH	7.0 ± 0.2	7.0 ± 0.2	7.0 ± 0.2	7.0 ± 0.2
(2) Other media				
	Nitrate and peptone	Ca Lactate (Waksman <i>et al.</i> )	Citrate (Giltay)	Glucose (Fred & Waksman)
KNO <sub>3</sub>	5	1	2	1
KH <sub>2</sub> PO <sub>4</sub>	0	0	2	0
K <sub>2</sub> HPO <sub>4</sub>	1	0.5	0	0.5
CaCl <sub>2</sub>	0.1	0	0.4	0.5
MgSO <sub>4</sub>	0.2	0	2	0
FeCl <sub>3</sub>	0.02	Tr.	Tr.	0
Asparagine	0	0	2	0
Peptone	0.1-1.5	0	0	0
Ca lactate	0	10	0	0
Na citrate	0	0	17	0
Glucose	0	0	0	10
pH	7.2 ± 0.2	—	7.0	7.0

Calcium lactate medium: Waksman, Reuszer, Carey, Hotchkiss & Renn (1933).

Citrate (Giltay): Medium no. 55 in *Laboratory Manual of General Microbiology*, by E. B. Fred & S. A. Waksman (1928).

Glucose: medium no. 56 in the same book.

Tr. = trace.

In all, 32 isolations of denitrifying bacteria were made; 20 of these are described in this paper (see Table 2).

*Characteristics of the denitrifiers.* All the denitrifying bacteria that we were able to isolate appeared to be *Pseudomonas* species. They differed from most other described species of this genus in their ability to grow abundantly in saline media, and at 37°. All of them seemed to need a certain minimum amount of organic matter for denitrification. Increasing amounts of peptone were added to a mineral salt medium containing 0.5 % potassium nitrate; with 1500 p.p.m. peptone all the cultures showed signs of denitrification, but only three isolates were able to denitrify with 1000 p.p.m., though all the cultures could grow with this amount of peptone. Smaller amounts of peptone would support neither growth nor denitrification.

Hydroxylamine, which is a possible intermediate product of nitrate reduction (Tanaka, 1953), was toxic to all the isolates. They all grew in broth containing 0.005 % hydroxylamine, but only one culture (group D) would grow in 0.025 %; it did not, however, produce gas at this concentration.

Table 2. *Source of denitrifying Pseudomonas cultures*

Group	No. of isolates	Source	Method of isolation	Presumed identity
A	9	Sea water off Calicut	Sea-water agar plates	<i>P. marinodenitrificans</i> n.sp.
	2	Sea-water off Tuticorin	Sea-water agar plates	<i>P. marinodenitrificans</i> n.sp.
	1	Marine sand off Tuticorin	Waksman's medium with lactate	<i>P. marinodenitrificans</i> n.sp.
	2	Sea water off Calicut	Freshwater agar plates	As above, but able to grow in fresh-water media
B	1	Mussels	Sea-water agar	<i>P. mytili</i> n.sp.
C1	1	Mussels	Sea-water agar	<i>P. aeruginosa</i>
C2	1	Mussels	Sea-water agar	<i>P. aeruginosa</i>
D	2	Sea water off Calicut	Sea-water agar at 4°	<i>P. denitrificans</i>
E	1	Mussels	Sea-water agar	<i>Pseudomonas</i> sp.

Nitrite in 0.2 % concentration was decomposed completely in 48 hr. by all cultures except one (group D). In all cultures except two (groups D and B) gas production was noticed in the presence of 0.5 % sodium nitrite. Only one culture, *Pseudomonas aeruginosa* (group C2) decomposed nitrates with glucose as source of energy and produced gas from the former.

In all cases denitrification took place more rapidly under anaerobic conditions. In open test tubes 0.2 % potassium nitrate was decomposed to gas in 7 days, but the same amount in anaerobic tubes showed gas formation 48–72 hr. after inoculation, and nitrite had completely disappeared in 5 days. The isolates fell into one of five groups (see Table 2).

*Group A* consisted of 14 strains. Two of them differed from the rest in being able to grow in non-saline media. As will be seen in Table 3, this group is not able to denitrify large amounts of nitrate, for in 1 % nitrate broth it forms nitrite and small amount of gas only; but 0.2 % of nitrate in broth is reduced to gas. The strains in this group can reduce 0.2 and 0.5 % of sodium nitrite to gas; they form gas on Waksman's calcium lactate medium, but do not grow either on Giltay's medium (nitrate, citrate and asparagine) or on the very simple glucose-nitrate medium of Fred & Waksman (see Table 1). As the strains in this group differ from any previously described marine *Pseudomonas* species, we propose to include them in a new species, *P. marinodenitrificans*. The characteristics of this species are given in the Appendix.

*Group B* consisted of a single culture, isolated from mussels. It was capable of growth in non-saline media, and was a more energetic denitrifier than

group A. It reduces 1 % nitrate broth with larger gas volume; 0.2 % nitrate in broth is reduced completely to gas. Though 0.2 % sodium nitrite was reduced to gas, 0.5 % sodium nitrite inhibited gas production. Gas was slowly formed from Waksman's medium and more vigorously from Giltay's medium. But in the glucose + nitrate medium neither growth nor gas production was noted. This is one of the versatile denitrifiers utilizing a large number of amino acids for energy, in contrast to group A (Venkataraman & Sreenivasan, 1955). This culture differs from any hitherto described in the literature, and it is proposed to create for it a new species, *Pseudomonas mytili*. A full description of this is given in the Appendix.

Table 3. *Growth and reactions of groups of strains on different media*

Media	Group					
	A	B	C1	C2	D	E
1 % nitrate broth	Nitrite small gas volume	Gas	Gas	Nitrite small gas volume	Nitrite small gas volume	Gas
0.2 % nitrate broth	Gas	Gas	Gas	Gas	Gas	Gas
	(Reduction takes place in 7 days aerobically, in 2-3 days anaerobically)					
0.2 % nitrite broth	Gas	Gas	Gas	Gas	No reduction: slight growth	Gas
0.5 % NO <sub>2</sub> , 0.2 % NO <sub>3</sub> broth	Gas	No reduction: slight growth	Gas	Gas	No growth	Gas
Ca lactate and nitrate	Gas	Gas	Gas	Gas	No reduction: slight growth	No growth
Citrate and nitrate	No growth	Gas	Gas	Gas	Gas	Gas
Glucose and nitrate	No growth	No growth	No growth	Gas	No growth	No reduction: slight growth

Incubated at 37° (group D at room temperature, 28-30°).

## DISCUSSION

The known denitrifying bacteria can be classified in three genera: *Micrococcus* (Kluyver, 1953; Robinson & Gibbons, 1952); *Denitrobacillus* (Verhoeven, 1952); and *Pseudomonas*. Several denitrifying *Pseudomonas* species are known to exist in soil (Christensen, 1903; Meiklejohn, 1940); the most adequately described of these soil forms is *P. stutzeri* (van Niel & Allen, 1952); *P. aeruginosa* and *P. ureae* are also denitrifiers (Bergey's Manual, 1948).

But the evidence for the occurrence of denitrifying pseudomonads in the sea is not very clear. There are 13 species of this genus listed as 'sea water to brine' inhabitants in *Bergey's Manual*; the Manual does not mention that any of them produce gas from nitrate, but ZoBell & Upham (1944) say that two of them, *Pseudomonas calcis* and *P. calciprecipitans*, are active denitrifiers. The same authors isolated two new species of marine denitrifiers, *P. azotogena* and *P. perfectomarinus*. Lloyd (1931) described in detail a marine denitrifying organism which she called *Bacillus costatus* (*Vibrio costatus*). Recently, Sreenivasan (1956) has described some marine denitrifiers tolerating high concentrations of copper.



In the present paper we describe 20 isolates, which all reduce nitrate to gas, and all of which we consider to be strains of *Pseudomonas* spp. One isolate we have been unable to allot to a species; others belong to the known species *P. denitrificans* and *P. aeruginosa*; and finally there are several isolates which we consider should be classified in two new species, *P. marinodenitrificans* and *P. mytili*.

It thus appears that denitrifying bacteria are common in the sea off the coasts of South India; this is in striking contrast to the waters of the Gulf of Maine (Waksman, Carey & Reuszer, 1933; Waksman, Hotchkiss & Carey, 1933; Waksman, Reuszer, Carey, Hotchkiss & Renn, 1933). Waksman and his colleagues think that denitrification is not possible at the surface of open seas, as do Thompson & Gilson (1937). These two authors point out that an abundant source of easily oxidizable organic matter is necessary for denitrification, and they suppose that this is not obtainable in the waters of open seas. But ZoBell (1947) cites instances of heterotrophic bacteria in the euphotic zone which get much of their food from organic substances secreted by phytoplankton. Waksman, Carey & Reuszer (1933) noted that *Fucus* material may be used as a source of energy by denitrifying bacteria. Kadota (1951) showed that agar was oxidized by *Vibrio purpureus* during denitrification of potassium nitrate, and symbiotic denitrification was reported by Burgwitz (1935). It thus seems possible that plankton excreting organic matter, decomposing plankton, or other micro-organisms, may provide the organic matter necessary for bacteria to carry out denitrification in the sea. The ubiquitous nature of denitrifiers in marine materials is indeed interesting.

It is with pleasure we thank Professor C. E. ZoBell, Scripps Institution of Oceanography, for his keen interest in this work and for valuable suggestions and criticism. This paper is published with the permission of the Director of Industries and Commerce (Fisheries), to whom our thanks are due.

## APPENDIX

### Characteristics\* of the new species of denitrifying bacteria

	Group A	Group B
Morphological characteristics	Straight rods, $0.4-0.6 \times 0.8 \mu$ , single and in pairs, actively motile with a single polar flagellum, Gram-negative, non-sporing, non-capsulated	Rods, straight, $0.5 \times 0.8 \mu$ , single and in pairs, actively motile with a single polar flagellum, Gram-negative, non-sporing
Cultural characteristics		
Agar colonies	Circular, grey, translucent flat, smooth, undulate margin	Grey, effuse, thin, smooth circular colonies: entire
Agar stroke	Bluish grey, echinulate, glistening, smooth, adherent, moderate growth	Grey, translucent, moist, glistening, smooth, becoming butyrous; abundant growth
Broth	Heavy turbidity and fragile membranous pellicle and ring. No fluorescent pigment	Uniform turbidity and pellicle but no fluorescent pigment

\* All media made up in 'aged' sea water.

## APPENDIX (cont.)

	Group A	Group B
Biochemical properties	Glucose, sucrose and lactose not fermented; starch not hydrolysed; gelatin not liquefied; litmus milk unchanged; indole and H <sub>2</sub> S not produced; nitrates reduced to nitrites and gaseous nitrogen; ammonia not produced from peptone; no growth on potato and on freshwater media without salt; grows on 10 % NaCl agar; good growth at 37° and at room temperature (28–32°). No fluorescent pigment. Aerobic, facultative	Acid from glucose, sucrose, maltose, adonitol and sorbitol, but none from glycerol and lactose. Gelatin not liquefied; litmus milk coagulated but not digested; indole and H <sub>2</sub> S (peptone iron agar) not produced; nitrates and nitrites vigorously reduced to gaseous nitrogen; fats hydrolysed; grows in fresh water media also; good growth at 37°; no chromogenesis. Aerobic, facultative
Source	Offshore sea water off Calicut and Tuticorin, and marine sand off Tuticorin	Green mussels ( <i>Mytilus edulis</i> ) in inshore sea off Calicut
Name proposed	<i>Pseudomonas marinodenitrificans</i> n.sp.	<i>Pseudomonas mytili</i> n.sp.

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## The Dissimilation of Amino Acids by *Rhodospirillum rubrum*

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**SUMMARY:** Washed suspensions of a strain of *Rhodospirillum rubrum* S1 grown anaerobically in the light and incubated in the light under argon metabolized many of the 15 amino acids tested and produced ammonia and carbon dioxide as the principal extracellular products. Hydrogen was also produced in the presence of glutamic acid. Of the glutamic acid metabolized 80 % of the carbon and 55–70 % of the nitrogen were converted into intracellular products. The magnitude of the catabolism of the other amino acids, as measured by ammonia and carbon dioxide production, appears to be an inverse function of the closeness to which the elemental composition of the amino acid approximates to that of cell material.

When the organism was grown aerobically in the dark, studies of the metabolism of amino acids by washed suspensions, under aerobic conditions, were complicated by the relatively high rate of endogenous respiration. In the presence of certain amino acids this was decreased by 9 % (with alanine) to 45 % (with aspartic acid), and glutamic acid, aspartic acid and alanine, at least, were oxidized to completion by the organism.

Although *Rhodospirillum rubrum* can utilize amino acids as sole hydrogen donor and nitrogen source for growth, no systematic studies on its amino acid metabolism have been published as far as the author is aware. The work described in this paper is the first part of an attempt to elucidate the mechanism of amino acid metabolism by washed suspensions of the organism when grown under anaerobic conditions in the light or under aerobic conditions in the dark. Hereafter suspensions grown under these two conditions will be referred to as 'light-grown organisms' and 'dark-grown organisms' respectively.

### METHODS

**Organism.** The organism used in the studies with dark-grown organisms was *Rhodospirillum rubrum*, strain S1, kindly supplied by Dr S. R. Elsdén. For the experiments with light-grown organisms a mutant strain which arose spontaneously was used. This differed from the parent in that it had a high rate of endogenous gas production and produced hydrogen in the presence of glutamic acid. It was maintained by weekly transfers in stab culture on 2 % (w/v) agar plus 0.3 % (w/v) Difco Yeast Extract, growth occurring in the light.

**Growth conditions.** The medium used throughout this work was based on that described by Kohlmeier & Gest (1951), and contained per litre: DL-malic acid, 6 g.; L-glutamic acid, 2 g.;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 mg.; biotin, 10  $\mu\text{g}$ .; Difco Yeast Extract, 600 mg.; trace element solution, 1.0 ml.; mineral salts solution ( $\text{K}_2\text{HPO}_4$ , 50 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 20 g.;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 4 g.;  $\text{m-H}_3\text{PO}_4$ , 400 ml.; per litre of solution), 10 ml.; the pH value was adjusted to 6.7 with NaOH before



autoclaving. Inoculations of the growth vessels were with 0.1 % (v/v) of a culture which had been grown in the light in 5 ml. of the above medium in a cotton-plugged test tube for 48 hr.

Light-grown organisms were cultured in a completely full reagent bottle and grown for  $3\frac{1}{2}$  days at  $30^\circ$  in an incubator illuminated by three 40 W. tungsten filament lamps at a distance of 25 cm. The organisms were harvested at a density of about 0.8 mg. dry wt./ml. Dark-grown organisms were grown for  $3\frac{1}{2}$  days in the dark at  $30^\circ$  in Roux bottles containing 150 ml. of medium.

Washed suspensions were prepared by harvesting and washing twice with 0.067 M-phosphate buffer (pH 7.0) containing 0.03 %  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ . The cells were finally suspended in this buffer at a density of 10–40 mg. dry wt./ml. Dry weights were determined by drying a sample to constant weight at  $105^\circ$  and applying a correction for the weight of salt present in the suspending medium.

*Manometric techniques.* Most of the experiments described were carried out in double side bulb Warburg manometers at  $30^\circ$ . In each experiment 1.0 ml. of the suspension was placed in the main compartment of the vessel, together with 1.0 ml. of 0.1 M-phosphate buffer and 0.5 ml. of substrate added from a side bulb after a 10–15 min. period of equilibration in the dark. To obtain consistent results in experiments with light-grown organisms it was necessary to avoid delay in dispensing the suspension, and in gassing and equilibrating the manometers. For the experiments performed in the light, the manometers were illuminated from below by a bank of eight 60 W. lamps which were turned on immediately after the addition of the substrate. In the dark experiments each vessel was covered with a small black bag. The reactions were terminated by the addition of 0.5 ml.  $\text{N-H}_2\text{SO}_4$  except in experiments involving total nitrogen determinations on the supernatant fluids and those to determine intracellular products of glutamic acid dissimilation.

In manometric experiments, where the gas phase was argon and a KOH paper was present, any gas evolved was taken to be hydrogen. Proof that this was so depends on the fact that it was absorbed by palladized asbestos plus methylene blue (Gest, Kamen & Bregoff, 1950) and that little or no gas was produced in the presence of both hydrogen and carbon dioxide absorbers. Thus hydrogen and carbon dioxide were apparently the only gases produced during metabolic experiments.

In the absence of a KOH paper both hydrogen and carbon dioxide were evolved. The amounts of the two gases were measured in the following manner. The reaction was carried out in a Warburg manometer with two side arms, one of which contained the substrate and the other 0.5 ml.  $\text{N-H}_2\text{SO}_4$  and also a sealed bulb containing 0.03 ml. 20 % NaOH. This bulb could be crushed by rotating the stopper, the end of which had been lengthened to extend into the side bulb. When the experiment was completed, the acid was tipped in from the side arm to release any combined carbon dioxide, and the total pressure change due to hydrogen plus carbon dioxide measured. The bulb containing the NaOH was then crushed and the carbon dioxide absorbed. The change in pressure was measured, and from the two readings the volumes of hydrogen

and carbon dioxide evolved during the reaction were calculated. Correction for the carbon dioxide initially present was made by running a parallel manometer in which the acid was tipped and the bulb containing NaOH crushed immediately after equilibration.

Respiratory quotients (R.Q.) were measured by the three vessel technique described by Umbreit, Burris & Stauffer (1949). Good agreement for ammonia production and amino acid disappearance was found between the vessels with and without KOH paper, showing that the amino acid metabolism was not seriously affected by removal of carbon dioxide.

At the end of the experiment the contents of each manometer vessel were washed out, made up to 10 ml. with water and the bacterial cells centrifuged off. Samples of the supernatant fluids were then used for total nitrogen, ammonia and amino acid determinations.

*Analytical methods.* Total nitrogen in the supernatants was determined by a micro-Kjeldahl method using the catalyst described by Chibnall, Rees & Williams (1943). The ammonia was estimated spectrophotometrically using Nessler's reagent after distillation in a Markham microdistillation apparatus (Markham, 1942) from a strongly alkaline solution. Free ammonia in suspension fluids was estimated similarly. When glutamine was present the pH value was adjusted to 9.3 and the ammonia distilled off by the method of Parnas & Heller (1924). L-Glutamic acid and L-glutamine were estimated together, using the specific L-glutamic acid decarboxylase of *Clostridium welchii* SR 12 (Gale, 1945). Other amino acids were estimated by the photometric ninhydrin method of Cocking & Yemm (1954) after the removal of ammonia by heating 1.0 ml. of the solution with 0.2 ml. N-NaOH on a boiling water-bath for 20 min. followed by neutralization with 0.1 N-H<sub>2</sub>SO<sub>4</sub>. The ninhydrin method gave values for L-glutamic acid which agreed well with those found by the glutamic decarboxylase method and this has been taken as evidence that, during the course of their metabolism, the organisms produced no compounds interfering with the ninhydrin estimation of amino acids.

*Measurement of radioactivity of carbon dioxide respired by <sup>14</sup>C-labelled organisms.* The method was based on that developed by Plackett (personal communication) using a Conway unit (Conway, 1950). The organisms were allowed to metabolize in the outer compartment of a Conway unit and the carbon dioxide evolved was trapped by 0.2 ml. saturated Ba(OH)<sub>2</sub> on a polythene disk in the centre compartment. At the end of the experiment the solution on the disk was dried *in vacuo*, and the <sup>14</sup>C estimated, using a thin mica end-window GM tube. A drop of indicator added to the disk ensured that the capacity of the baryta had not been exceeded. As the experiments were performed at pH 7.0 it was necessary to liberate dissolved carbon dioxide. To do this a small piece of glass tubing of 2 mm. internal diameter, sealed at one end and containing 0.05 ml. 10 N-H<sub>2</sub>SO<sub>4</sub>, was attached to the ground glass lid of the Conway unit with sealing wax; at the end of the experiment this tube was dislodged and the acid mixed with the contents of the outer compartment. One hour was then allowed for the absorption of the liberated carbon dioxide by the baryta. The experiments were begun by the addition of 1.0 ml. of bacterial suspension to 0.8 ml.

0.1 M-phosphate buffer and 0.5 ml. substrate in the outer compartment, which was then sealed. A unit in which the acid was added immediately after the bacterial suspension served as a control for the amount of  $^{14}\text{CO}_2$  present initially. Each estimation was performed in triplicate.

$^{14}\text{CH}_3.^{14}\text{COONa}$  was supplied by the Radiochemical Centre, Amersham.

## RESULTS

### *The metabolism of amino acids by light-grown organisms*

Washed suspensions of *Rhodospirillum rubrum*, when incubated under argon in light, metabolized many of the amino acids tested (Table 1). The rates of disappearance and ammonia production from each amino acid were compared to L-glutamic acid. The short chain and most highly oxidized amino acids were metabolized most rapidly. Hydrogen was not produced during the photo-metabolism of any of the amino acids with the exception of glutamic acid and of proline, the latter increasing the hydrogen production above that of the endogenous value only when the endogenous production was occurring rapidly.

Table 1. *Metabolism of amino acids by light-grown organisms*

Experiments carried out in Warburg flasks under argon in light at 30° in the absence of KOH paper with bacteria suspended in 0.067 M-phosphate buffer pH 7.0. The rate of amino acid disappearance was compared in each experiment to that of L-glutamic acid. The results are a summary of those from several experiments.

Amino acid	Hydrogen production	Relative rate of amino acid disappearance (L-glutamic acid = 100)	$\mu\text{mole NH}_3$ formed (less endogenous) per $\mu\text{mole}$ of amino acid disappearing
L-Aspartic acid	—	126	0.63
DL-Serine	—	123	0.87
L-Alanine	—	110	0.65
L-Glutamic acid	+	100	0.13–0.30
L-Glutamine	—	61	0.81
Glycine	—	53	0.74
DL-Threonine	—	49	0.60
L-Ornithine	—	33	.
L-Arginine	—	18	.
L-Proline	+*	13	.
L-Valine	—	13	.
L-Histidine	—	10	.
L-Lysine	—	9	.
L-Leucine	—	0	.
L-Hydroxyproline	—	0	.

\* Shown to increase the hydrogen production above that of the endogenous only when the endogenous production was occurring rapidly.

*The production of  $\text{H}_2$ ,  $\text{CO}_2$  and  $\text{NH}_3$  from amino acids.* Table 2 shows the production of hydrogen, carbon dioxide and ammonia during the metabolism of four of the amino acids. In the absence of a KOH paper, 15–20 % of the glutamic acid carbon appeared as carbon dioxide and 15–30 % of the nitrogen appeared as ammonia. In the presence of a KOH paper less than 5 % of the



nitrogen appeared as ammonia. Total nitrogen determinations showed that, in the absence of a KOH paper, an additional 12–15 % of the glutamic acid nitrogen was present as nitrogen-containing compounds other than ammonia. Small amounts (12  $\mu$ mole from 280  $\mu$ mole L-glutamic acid) of volatile fatty acids have been detected in the supernatant fluids. Experiments with  $^{14}\text{C}$  glutamic acid (not reported here) indicated that less than 3 % of the glutamic acid carbon which had disappeared from the medium could be accounted for as non-volatile products in the medium. These findings, taken in conjunction with the manometric results, indicate that 80 % of the carbon and 55–70 % of the nitrogen was assimilated by the organism.

Table 2. *The dissimilation of amino acids by light-grown organisms*

Experiments carried out in Warburg flasks under argon in light at 30°. Bacteria suspended in 0.067 M-phosphate buffer pH 7.0 at dry wt.: Expt. A, 30.0 mg.; Expt. B, 23.1 mg. Duration of experiment 3 hr.; 50  $\mu$ mole of amino acid added initially.

Amino acid	KOH paper present	H <sub>2</sub> formed ( $\mu$ mole)	CO <sub>2</sub> formed ( $\mu$ mole)	NH <sub>3</sub> formed ( $\mu$ mole)	Amino acid metabolized ( $\mu$ mole)
Expt. A					
None	+	9.3	.	0	.
	—	0	7.0	0.6	.
L-Glutamic acid	+	21.2	.	0.6	19.5
	—	6.8	24.6	3.7	24.3
L-Alanine	+	0	.	5.4	11.1
	—	0	10.8	18.1	26.8
Glycine	+	0	.	3.5	4.8
	—	0	7.9	10.2	12.9
Expt. B					
None	+	0.3	.	0.5	.
	—	0	5.2	1.6	.
L-Glutamic acid	+	10.8	.	0.9	8.0
	—	3.0	10.4	6.5	16.8
L-Aspartic acid	+	0	.	10.8	15.0
	—	0	34.8	14.9	21.2

Under parallel conditions in the dark no disappearance of L-glutamic acid from the medium was observed nor was the small endogenous carbon dioxide production increased by the presence of substrate.

*Distribution of other products of L-glutamic acid dissimilation.* In these experiments the contents of each manometer vessel were centrifuged, washed twice and finally resuspended in 0.85 % NaCl. For the estimation of free glutamic acid in the organism, the bacteria were disrupted by boiling as described by Gale (1947). For the estimation of total L-glutamic acid (combined plus free), the bacteria were hydrolysed in 6N-HCl in a sealed tube for 16 hr. at 105° and the hydrochloric acid removed by vacuum distillation. Cellular ammonia and amide nitrogen were estimated by hydrolysing the cells in N-H<sub>2</sub>SO<sub>4</sub> at 100° for 3 hr. according to the method of Pucher, Vickery & Leavenworth (1935), and determining the ammonia as described above.

Although *Rhodospirillum rubrum* takes up L-glutamic acid readily, no significant quantity has ever been found adsorbed on the outside of, or free inside,



the cell. Of the glutamic acid-nitrogen disappearing from the medium, 10-20 % could be accounted for as bound glutamic acid in the cell, while the increase in ammonia and amide groups accounted for an additional 25 % (Table 3). The remaining 55-65 % was not present as L-glutamic acid and probably had been metabolized further.

Table 3. *Distribution of some intracellular products of L-glutamic acid dissimilation by light-grown organisms*

Experiments carried out in Warburg flasks under argon in light at 30°. Each flask contained 35.7 mg. dry wt. bacteria suspended in 0.067 M-phosphate buffer pH 7.0. Duration of experiment 3 hr.; 50  $\mu$ mole L-glutamic acid added initially. Results expressed per 100 mg. dry wt. bacteria.

	Additions	
	Nil ( $\mu$ mole)	L-glutamic acid ( $\mu$ mole)
L-Glutamic acid metabolized	.	52.3
NH <sub>3</sub> formed	0.8	2.4
Change in cellular L-glutamic acid	-4.5	+5.0
Change in cellular 'ammonia'	-3.4	+11.3

Initial level of cellular L-glutamic acid: 43.7  $\mu$ mole.

Initial level of cellular 'ammonia': 59.3  $\mu$ mole.

*The effect of incubation in carbon dioxide, nitrogen and hydrogen.* The disappearance of glutamic acid over a 3 hr. period was the same under 5 % CO<sub>2</sub> + 95 % argon as under 100 % argon but the presence of a KOH paper decreased the rate. Further information on the role of carbon dioxide in glutamic acid metabolism was obtained from experiments performed in Krebs's vessels with nitrogen in the gas phase. Three Krebs's vessels were initially placed in the dark at 0° to decrease the metabolism. The vessels were then evacuated and filled, one with 5 % CO<sub>2</sub>-95 % N<sub>2</sub> and the other two with CO<sub>2</sub>-free nitrogen. One of these was then closed, and the other gassed with CO<sub>2</sub>-free nitrogen throughout the experiment. The disappearance of glutamic acid was greatest in an atmosphere enriched with CO<sub>2</sub>, suggesting that it was probably essential for glutamic acid uptake (Table 4).

Table 4. *Effect of carbon dioxide on L-glutamic acid disappearance in the presence of light-grown organisms*

Experiments carried out in Krebs's vessels in light at 30°. Vessel contained 47.1 mg. dry wt. bacteria suspended in 4.0 ml. 0.067 M-phosphate buffer pH 7.0. Duration of experiment 3 hr.; 50  $\mu$ mole L-glutamic acid added initially.

	L-glutamic acid disappearance ( $\mu$ mole)
Vessel gassed with 95 % N <sub>2</sub> + 5 % CO <sub>2</sub>	42.9
Vessel gassed initially with CO <sub>2</sub> -free N <sub>2</sub> and then closed	17.4
Vessel gassed with CO <sub>2</sub> -free N <sub>2</sub> initially and throughout the experiment	10.4

Replacement of argon in the gas phase by hydrogen or nitrogen did not alter the rate of glutamic acid disappearance although nitrogen, but not hydrogen, completely inhibited hydrogen production.

*The amino acid metabolism of dark-grown organisms aerobically in the dark*

Studies of amino acid metabolism by these organisms, under aerobic conditions in the dark, was made difficult by the high rate of endogenous metabolism. The rate of oxygen uptake with glutamic acid, the most actively attacked of the four amino acids tested, was never greater than twice the endogenous value. Previous aeration of a washed suspension of the organism decreased the endogenous respiration by 50 % but did not increase the proportion by which the oxygen uptake was raised on the addition of glutamic acid. All the experiments were therefore carried out with organisms harvested and washed by the usual procedures.

*The effect of substrate metabolism on the endogenous respiration.* The organisms incorporated  $^{14}\text{C}$  into their endogenous reserves during growth in the normal medium plus  $2.1\text{ }\mu\text{g./ml.}$  of doubly-labelled acetate. After harvesting, the cells were washed twice and then incubated in Conway units in the presence or absence of an excess of an amino acid. The amount of  $^{14}\text{CO}_2$  produced gives a measure of the magnitude of the endogenous respiration, assuming that the endogenous substrates have been uniformly labelled under these growth conditions. All the amino acids tested diminished the endogenous metabolism (Table 5). L-Aspartic acid had the greatest effect, decreasing it by 45 %, whereas

Table 5. *The effect of amino acids on the endogenous metabolism of dark-grown organisms*

The organisms were grown on the normal medium plus  $2.1\text{ }\mu\text{g./ml.}$  of sodium acetate doubly labelled with  $^{14}\text{C}$  and having a specific activity of  $48\text{ }\mu\text{C./mg.}$  Incubation was in Conway units aerobically in the dark at  $30^\circ$ . Outer compartment contained *c.*  $1.5\text{ mg.}$  dry wt. of bacteria suspended in  $0.067\text{ M-phosphate}$  buffer pH 7.0.  $^{14}\text{CO}_2$  respired was trapped by  $\text{Ba(OH)}_2$  on a polythene disk, dried down and the  $^{14}\text{C}$  estimated. Duration of experiment 3 hr.;  $20\text{ }\mu\text{mole}$  of amino acid added initially. Radioactivity of bacteria added to each vessel 10,600 counts/min.

Amino acid added	Radioactivity of $\text{CO}_2$ formed (counts/min.)	Relative rate of endogenous respiration based on radioactivity of respired $\text{CO}_2$
None	$295 \pm 12$	100
L-Glutamic acid	$173 \pm 12$	59
L-Aspartic acid	$163 \pm 28$	55
L-Alanine	$270 \pm 6$	91
Glycine	$251 \pm 16$	85

L-alanine decreased it by only 9 %. On the basis of these results, the carbon dioxide output and oxygen uptake values quoted in Table 6 have been corrected for the decreased endogenous metabolism in the presence of each amino acid. When these corrections were made the R.Q. values observed in the presence of the amino acids approached more closely the theoretical values for complete oxidation than did the uncorrected figures (Table 6). The results suggest that the amino acids were oxidized to completion.

Table 6. *Metabolism of L-glutamic acid, L-aspartic acid, L-alanine and glycine by dark-grown organisms*

Experiments carried out in Warburg flasks aerobically in the dark at 30°. Main compartment contained 13.1 mg. (Expt. A) and 18.5 mg. (Expt. B) dry wt. of bacteria suspended in 0.067 M-phosphate buffer pH 7.0. Duration of Expt. A 8½ hr., B 5 hr.; 10  $\mu$ mole of amino acid added initially. The corrected CO<sub>2</sub> output has been calculated by subtraction of the decreased endogenous CO<sub>2</sub> output in the presence of each amino acid (Table 5). A proportional decrease in oxygen uptake has been assumed.

Expt.	Amino acid added	Amount metabolized ( $\mu$ mole)	NH <sub>3</sub> ( $\mu$ mole)	CO <sub>2</sub> ( $\mu$ mole)		O <sub>2</sub> ( $\mu$ mole)		R.Q.		
				O.V.*	C.V.	O.V.	C.V.	O.V.	C.V.	T.V.
A	None	0	2.6	27.6	.	31.1	.	0.89	.	.
	Glycine	5.5	7.7	45.2	21.7	44.3	17.9	1.02	1.21	1.33
	L-Aspartic acid	10.0	10.5	58.2	43.0	51.2	34.1	1.14	1.26	1.33
	L-Alanine	9.0	8.4	43.1	18.0	46.8	18.6	0.94	0.97	1.00
B	None	0	1.6	47.3	.	50.7	.	0.93	.	.
	L-Glutamic acid	9.0	10.0	75.2	47.3	74.2	44.2	1.01	1.07	1.11

\* O.V. = observed value; C.V. = corrected value; T.V. = theoretical value.

### DISCUSSION

The carbon:nitrogen ratio of many micro-organisms is approximately 5:1 (Buchanan & Fulmer, 1928). The results suggest that the light-grown organisms form more ammonia per mole of substrate attacked from amino acids with C:N ratios less than 5:1 than they do from amino acids with a ratio similar to that of the organisms. For instance, only 15–30 % of the theoretical amount of ammonia was formed from glutamic acid (C:N ratio of 5:1), whereas 80 % of the theoretical amount was released during the metabolism of glycine (C:N ratio of 2:1). Similarly, the amount of carbon dioxide produced seems to be related to the state of oxidation of the amino acid. Thus with aspartic acid, which contains 48 % oxygen, more carbon dioxide was produced per mole of substrate disappearing than with alanine which contains only 36 %. There is a marked similarity between these results and those quoted by van Niel (1941) for the assimilation of carbon dioxide during the metabolism of fatty acids by this organism. He found that as the percentage of oxygen in the molecule decreased, the amount of carbon dioxide assimilated per molecule of substrate metabolized increased.

Further investigations with <sup>14</sup>C labelled amino acids are being carried out to determine into what cellular components the amino acid carbon is incorporated.

In Table 6 a comparison has been made between the R.Q. values for complete oxidation of glycine, alanine, aspartic acid and glutamic acid and the corrected observed R.Q. values of dark-grown organisms. In arriving at these observed R.Q. values two assumptions have been made: (a) that the R.Q. value for the endogenous metabolism does not change in the presence of the substrate so that a decrease of endogenous carbon dioxide production in the presence of an amino acid is accompanied by a proportional reduction in oxygen uptake; (b) that the decrease in endogenous metabolism remains constant

throughout the experiment. There is no evidence to support or disprove the validity of these assumptions, but if they are true it would appear that alanine, aspartic acid, glutamic acid and possibly glycine are oxidized almost to completion in the dark by dark-grown *Rhodospirillum rubrum*.

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## Nuclear Morphology of *Bacillus cereus* grown on Partially Defined Media

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**SUMMARY:** When *Bacillus cereus* was grown on a medium solidified with agar but of otherwise known composition (salts, glucose, urea) impression preparations made after 2½ hr. of growth at 30° showed simple round nuclei by the osmic acid-hydrochloric acid-Giemsa technique. Similarly treated preparations made after 24 hr. of incubation showed well-marked unstained refractile inclusions. These inclusions, whose lipid nature was indicated by staining with Sudan Black, were closely associated with nuclear material, often appearing to be surrounded by an unbroken ring, a granular band, or a horse-shoe shape of nuclear material. These observations support the suggestion of Delaporte (1950) that lipid inclusions may distort nuclear shapes. These distortions might be misinterpreted as mitotic figures.

The subject of the structure and mode of division of the bacterial nucleus is still controversial. In an attempt to bring a different approach to this problem, a study has been made of the way in which various nuclear patterns can be produced regularly in response to altered nutritional conditions.

### METHODS

The organism used throughout was a strain of *Bacillus cereus* (NCTC 8035). Stock cultures were maintained on nutrient agar. A washed suspension (0.1 ml.) of organisms grown for 18 hr. in meat-extract broth at 30° was spread over the surface of the following media solidified with agar: basal medium; basal medium + 1 % (w/v) glucose, or + 1 % (w/v) urea, or + 1 % (w/v) glucose and 1 % (w/v) urea; nutrient agar. The basal medium (modified from Meiklejohn, 1950) had the following composition: dipotassium hydrogen phosphate ( $K_2HPO_4$ ) 1 g., sodium chloride 2 g.,  $MgSO_4 \cdot 7H_2O$  0.5 g., ferric chloride, trace, calcium carbonate 10 g., agar 20 g., tap water 1000 ml. The final pH value of this medium was 7.6; it was sterilized at 15 lb./sq.in. for 15 min.

Impression preparations, made from the growths on all these solid media after 2½ and after 24 hr. of incubation at 30°, were treated by the osmic acid-hydrochloric acid-Giemsa technique (HCl-Giemsa) (Robinow, 1944) for the demonstration of nuclear material. Preparations 24 hr. old were also stained by Sudan Black for the demonstration of lipid material. Wet preparations were made and examined by the phase-contrast microscope. Preparations made after 34 hr. of growth on basal medium + urea + glucose were stained by HCl-Giemsa and also examined by phase-contrast microscopy.

Photographs were taken by a 'Laboratory' photomicrographic camera (W. Watson and Sons Ltd.) on Ilford rapid process panchromatic plates.

## RESULTS

When the organism was grown on basal medium + glucose + urea at 30° for 2½ hr. the nuclear material demonstrated by the HCl-Giemsa method had the form of a simple round structure, generally in the centre of the cell (Pl. 1, fig. 1*a*). The division of the nuclear material in this actively growing culture appeared to be simple, consisting of a constriction of the nucleus into two daughter nuclei (Pl. 1, fig. 1*b*). Occasionally there was elongation of the nucleus as well as constriction (Pl. 1, fig. 1*c*).

After 24 hr. of incubation, the arrangement of the nuclear material was considerably changed. There were a few simple nuclei but the majority of the organisms contained nuclei with a round unstained refractile area in their centres (Pl. 1, fig. 2*a*). These structures, i.e. the central unstained area and the surrounding nuclear material, were named X-structures. There was a range in size of these unstained areas from small to large; they also showed variety in distribution, some being single, others in groups of two (Pl. 1, fig. 2*b*) or three. The surrounding band of nuclear material also showed differences in shape; in some organisms it was a complete band (Pl. 1, fig. 2*c*), in some it was horse-shoe shaped (Pl. 1, fig. 2*d*) and in others it was granular (Pl. 1, fig. 2*e*).

Staining with Sudan Black revealed that visible lipid (Pl. 1, fig. 3) was present in the organisms grown on the basal medium + urea + glucose. These lipid structures showed a range in size from small to large; they also showed variety in their distribution, some being single, others in groups of two or three. In short, they were similar in size, shape and distribution to the unstained areas of the X-structures. In a given set of fields counts were made of the number of X-structures demonstrated by the HCl-Giemsa method and of the number of inclusions in the Sudan Black preparations. The two counts were very close: 41.2 X-structures and 42.7 lipid granules/100 organisms.

Wet preparations of the same 24 hr. growth examined by phase-contrast microscopy revealed dark round granules (Pl. 2, fig. 4). These granules had a range in size from small to large, and they also showed variety in their distribution, some being single, others in pairs, and others in groups of 3.

Phase-contrast microscopy of cultures grown on basal medium agar + urea + glucose for 34 hr. at 30° revealed that developing endospores had a characteristic appearance when examined by this method (Pl. 2, fig. 5). They were oval and very refractile and could not be mistaken for the dark granules illustrated in Pl. 2, fig. 4. Developing endospores also gave a characteristic appearance when impression preparations taken after 34 hr. growth were stained by the HCl-Giemsa method (Pl. 2, fig. 6). They were darkly staining oval structures without internal differentiation, and could not be mistaken for X-structures.

Neither lipid inclusions nor X-structures were seen when the organism was grown on: basal medium alone; basal medium + glucose; basal medium + urea; nutrient agar after 24 hr. growth at 30°.

## DISCUSSION

The observations of this paper show that the nuclear material of *Bacillus cereus* (NCTC 8035) can be distorted into bizarre and unusual shapes by lipid inclusions and that these distorted nuclear forms can be produced by growing the organisms on a suitable medium. These observations support the suggestion of Delaporte (1939, 1950) that lipid and other inclusions may alter the shape of the bacterial nucleus, producing structures which in Delaporte's words are 'not determinate ones but altered passive ones'. The possibility of taking nutritional factors into consideration in the interpretation of results may well deserve more attention from bacterial cytologists. Perhaps the mitotic figures described by DeLamater & Mudd (1951) in *Bacillus megaterium* were no more than simple nuclei distorted by inclusions composed of lipid. The present findings emphasize the need for more and different approaches to the study of the bacterial nucleus. Not only must techniques for the demonstration of nuclear material be correlated with studies of living unstained and unfixed organisms, but the results need integration with the demonstration of cell inclusions, especially lipid inclusions.

I should like to express my indebtedness to the Rankin Research Fund of Glasgow University; to Mrs V. McKenzie, B.Sc., who greatly assisted me in the early stages of this investigation; and to Mr G. Kerr of the Pathology Department of the University of Glasgow for taking the negative of Pl. 1, fig. 2.

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## EXPLANATION OF PLATES

*Bacillus cereus* NCTC 8035 grown on basal medium agar+1% glucose+1% urea at 30°.

## PLATE 1

- Fig. 1. Actively growing culture (2½ hr. old) stained by the HCl-Giemsa method showing: (a) round nucleus; (b) constriction of dividing nucleus; (c) elongation and constriction of nucleus. (×3000)  
Fig. 2. X-structures. 24 hr. culture stained by the HCl-Giemsa method showing many nuclei with clear central areas as in (a). These clear areas may be single or in groups of two as in (b). Nuclear material is sometimes a complete band (c), horse-shoe shaped (d) or granular (e). (×3000)  
Fig. 3. 24 hr. culture stained by Sudan Black, showing lipid inclusions which show the same variety in size and distribution as the clear areas of the X-structures in fig. 2. (×2500)

## PLATE 2

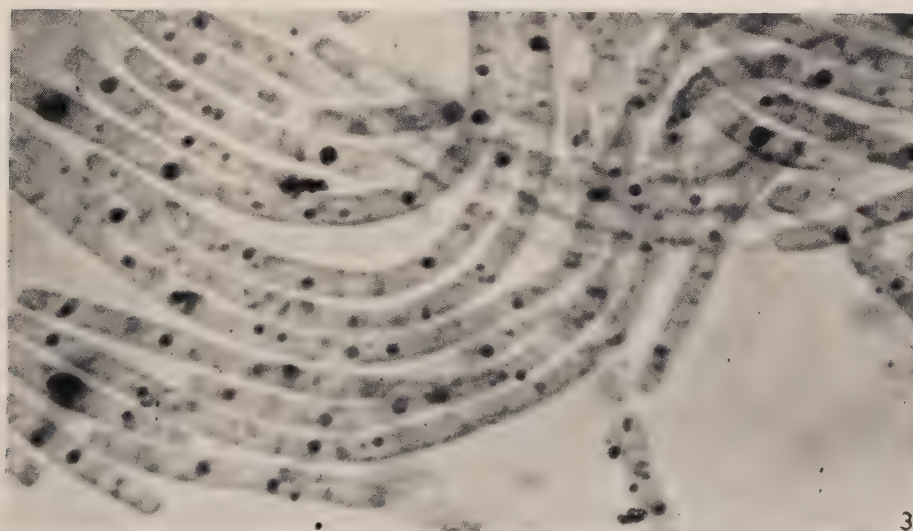
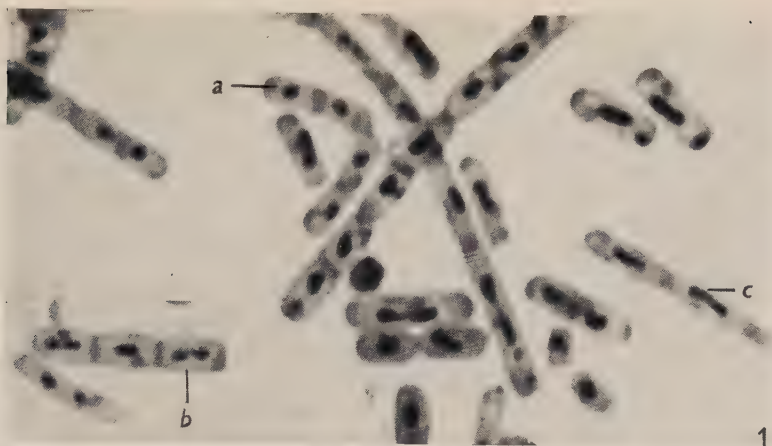
Fig. 4. 24 hr. culture examined by phase-contrast microscopy, showing round dark granules of different sizes and with a varied distribution. ( $\times 2500$ )

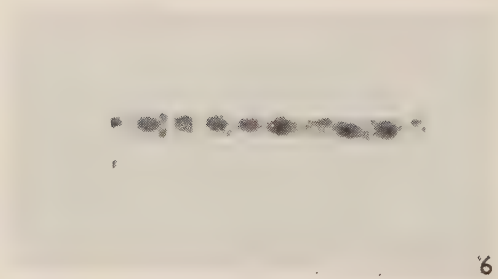
Fig. 5. Developing endospores as demonstrated by phase-contrast microscopy in a 34 hr. culture. ( $\times 2000$ )

Fig. 6. Darkly-staining developing endospores demonstrated by the HCl-Giemsa method in a 34 hr. culture. ( $\times 2000$ )

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## Non-identity of the Phospholipase of *Bacillus anthracis* with the Anthrax Toxin

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**SUMMARY:** Filtrates from suitable cultures of *Bacillus anthracis* contain a phospholipase which slowly hydrolyses the phospholipid in egg-yolk broth but has no action on free egg-lecithin. This enzyme does not appear to be the anthrax toxin.

McGaughey & Chu (1948) showed that *Bacillus anthracis* produced an opalescence when growing in a medium containing egg yolk, but the effect was less than that produced by *B. cereus* and *B. mycoides*. *B. anthracis* growing in the blood of infected guinea-pigs produces an extracellular toxin which kills the host in secondary shock (Smith, Keppie, Stanley & Harris-Smith, 1955; Smith, Keppie & Stanley, 1955); a lethal amount of this toxin is only produced by the growth of a large number of organisms in the blood during the terminal phase of anthrax. Lecithinases, responsible for the egg-yolk reaction of certain bacteria, are involved in their toxigenicity (MacFarlane, 1955) and when injected can produce the symptoms of shock (Berg, Levinson & Wang, 1951). A possible relationship between the egg-yolk reaction and the toxin of *B. anthracis* has therefore been investigated. First, the egg-yolk reaction was examined to see whether the opalescence produced by *B. anthracis* was due to an extracellular product of the organism and whether this product was a lecithinase. Then, the toxic plasma of infected guinea-pigs was investigated for the presence of such an enzyme and for a connexion between it and the toxic action.

### METHODS

**Organism.** A spore suspension of *Bacillus anthracis*, strain N.P., as used in all previous work (Smith *et al.* 1955) was the source of organisms.

**Egg-yolk broth (E.Y.B.).** Two egg yolks/l. 1% peptone solution containing 0.5% NaCl and at pH 7.4.

**Plasma containing the anthrax toxin.** This was collected from guinea-pigs dying of anthrax as described by Smith *et al.* (1955).

**Anthrax antiserum.** This was prepared in the horse by injecting the 'Sterne' strain of *Bacillus anthracis* (Belton & Strange, 1954).

**Test for phospholipase activity.** A sterile mixture of the solution to be tested (2 ml.) and E.Y.B. (2 ml., containing c. 50  $\mu$ g. ether-soluble P) was incubated at 37° and pH 7.4. After cooling the mixture, it was extracted with ether (3 ml.) and the P content of the ethereal extract determined by the method of Fiske & SubbaRow (1925). Activities are expressed as the percentage decrease in the ether-soluble P produced by the test materials in a stated time. Controls incubated for 72 hr. at 37° did not show a decrease in ether-soluble P.



## RESULTS

*The egg-yolk reaction with the growing organism*

E.Y.B. was inoculated with *Bacillus anthracis* and incubated at 37° for 4 days. The culture was then opalescent; centrifugation deposited the bacteria and produced a layer of fat on top of the clear medium. In three experiments the ether-soluble P of the medium was decreased 90 % by the growth of the organism.

*Culture filtrates with phospholipase activity*

*Preparation.* Media (E.Y.B., 1 % peptone or tryptic meat broth; 100 ml.) were inoculated with  $6 \times 10^7$  spores and incubated at 37° and pH 7.4 in a flask (250 ml.) for 2 days. After filtration through 'Millipore' filters (Lovell Chemical Co., Mass., U.S.A.) the sterile filtrate was stored at 0°.

*Selection of medium.* Table 1 shows the results of tests for phospholipase activity on filtrates from cultures of *Bacillus anthracis* in E.Y.B., 1 % peptone and tryptic meat broth. Filtrates from cultures in E.Y.B. and 1 % peptone were consistently active but cultures in tryptic meat broth T.M.B. had a much smaller and variable activity. Filtrates from cultures in 1 % peptone were used in the following work.

Table 1. *Phospholipase activity of culture filtrates from different media*

Culture medium		Phospholipase activity*	
		Decrease (%) in ether soluble P during	
		24 hr.	48 hr.
E.Y.B.	(1)	61	81
	(2)	83	90
Peptone	(1)	90	95
	(2)	79	90
T.M.B.	(1)	6	9
	(2)	14	34

\* For details of test for phospholipase activity see text.

*Activity.* Table 2 shows the slow progressive decrease with time of the ether-soluble P of E.Y.B. when the culture filtrate was examined in the test for phospholipase activity.

*Effect of pH.* In 8 hr. a culture filtrate decreased the ether-soluble P of E.Y.B. by 10, 14, 35, 35 and 18 % when examined at values of pH 5.6, 6.7, 7.4, 8.0 and 8.8, respectively, in the test for phospholipase activity.

*Effect of calcium.* The mixture of E.Y.B. and peptone culture filtrate used in the phospholipase test contained *c.* 0.001 M-Ca<sup>++</sup>. Increase of the Ca<sup>++</sup> concentration to 0.003, 0.006, 0.01 and 0.05 M did not increase the phospholipase activity, although addition of a high concentration of sodium citrate (0.1 M) decreased it.



*Stability.* There was no loss in phospholipase activity on storage at 0–4° for several weeks. No appreciable loss in activity occurred after heating the culture filtrate to 60° for 1 hr., but heating at 100° for the same period destroyed the activity. After shaking with ballotini beads and air for 16 hr. at 0–4° the activity was decreased about 50 %.

Table 2. *The progressive decrease of ether soluble P when a peptone culture filtrate was incubated with egg-yolk broth at pH 7.4 and 37°*

Time of incubation (hr.)	Decrease (%) of ether soluble P
4	9
8	26
12	47
16	53
18	66
21	76
24	79
32	88
41	98

*Specificity.* Active culture filtrates did not liberate inorganic phosphate from solutions (0.1 %) of sodium  $\alpha$ - and  $\beta$ -glycerophosphates or adenylic acid. Sodium monophenyl phosphate was slowly hydrolysed; under the conditions of the test for phospholipase activity in which the E.Y.B. was replaced by a solution (0.1 %) of sodium monophenyl phosphate, the P set free as inorganic phosphate in 72 hr. (about 15 % of the total P) was far less than the decrease of ether-soluble P in a normal phospholipase test.

The culture filtrates did not hydrolyse either lecithin (egg) or cephalin (sheep brain). The tests were carried out as in the test for phospholipase activity, i.e. at pH 7.4 and 37° for 48 hr. with E.Y.B. replaced by aqueous emulsions (0.2 %) of lecithin or cephalin. The addition of  $\text{Ca}^{++}$  (0.002 M and 0.005 M) did not affect hydrolysis of the lecithin. No haemolysis or increase in fragility of guinea-pig erythrocytes was produced when they were incubated with culture filtrates for 24 hr. at 37°.

Table 3. *The effect of anthrax antiserum on phospholipase activity*

Material	Phospholipase activity	
	Decrease (%) in ether soluble P during 24 hr.	
	No addition	With antiserum (0.3 ml.)*
Control of normal plasma (0.3 ml.)	8	19
Toxic plasma (0.3 ml.)	98	100
E.Y.B. culture filtrate (2 ml.)	91	98
Peptone culture filtrate (2 ml.)	82	95

\* This quantity of antiserum neutralized 9 ml. of toxic plasma (Smith, Keppie & Stanley, 1955).

*Absence of a connexion between the phospholipase and the anthrax toxin*

The active culture filtrates did not produce oedema when injected into the skin of a guinea-pig as did the toxic plasma of guinea-pigs dying of anthrax. The toxic plasma had a higher phospholipase activity than the non-toxic culture filtrates; 0.3 ml. of toxic plasma had approximately the same activity as 2 ml. of culture filtrate. However, Table 3 shows that neither the phospholipase activity of the toxic plasma nor that of the culture filtrates was inhibited by an amount of anthrax antiserum which was in excess of that required to completely neutralize the toxic action of the plasma.

## DISCUSSION

McGaughey & Chu (1948) could not detect an enzyme responsible for the egg-yolk reaction in culture filtrates of *Bacillus anthracis*. Contrary to this, we have found that filtrates from cultures in suitable media do contain such an enzyme, although the activity is small. The enzyme did not attack purified egg-lecithin. The hydrolysis of phospholipid by the enzyme may be dependent on the 'combined' state of the substrate in E.Y.B. It is relevant to point out here that lecithinase preparations from *Bacillus cereus* and *Clostridium welchii*, which hydrolysed equal amounts of free phospholipid, differed greatly in their ability to hydrolyse lipovitellenin (Chu, 1949). The phospholipase of *B. anthracis* resembles bacterial lecithinases in being fairly stable to heat and sensitive to surface denaturation.

Plasma containing the anthrax toxin also contains a phospholipase, but the two are probably not connected since anthrax antitoxin did not inhibit the phospholipase activity of the toxic plasma or of culture filtrates. In the case of lecithinase preparations from *Clostridium welchii* and *Bacillus cereus*, both the egg-yolk reaction and the toxicity are inhibited by the same homologous antiserum (MacFarlane, 1955; Chu, 1949).

This work was part of a thesis submitted by one of us (H.T.Z.) for the Ph.D. degree of the University of London.

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## Variations in a Related Series of Staphylococcal Bacteriophages

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**SUMMARY:** Stocks of staphylococcal phage 47C (serological group A) contained some group B phage. This was found to have originated in a lysogenic staphylococcus previously used to propagate phage 47C and which had subsequently lost its lysogenicity. The lysogenic phage was thus perpetuated in the phage stocks as a lytic phage. The characters of the two phages are described. When they lysogenized five different strains of staphylococci changes in typing pattern were produced. There was evidence which indicated that, in the prophage state, the two phages occupy different sites and that there is no cross-immunity between them. The propagation of the phages in different hosts resulted in changes in their host range. A virulent mutant of the B prophage was induced by the application of a variety of phages to the strain carrying it.

Since the introduction of staphylococcal phage typing (Fisk, 1942; Wilson & Atkinson, 1945) one method of preparing new phages has been by 'adaptation'. A staphylococcus not lysed by any of the typing phages at their routine test dilution may show plaques when tested with an undiluted phage; the phage thus revealed can then be propagated to high titre on the previously untypable strain. This 'adapted' phage may be a lysogenic contaminant derived from the strain used to propagate the original phage, and thus have no relationship to the phage from which it has been 'adapted'. Such was the origin of phage 42C (Rountree, 1949*a*). Since nearly all strains of *Staphylococcus aureus* are lysogenic (Rountree, 1949*b*), contaminating lysogenic phage may be the origin of many 'adapted' phages. However, the behaviour of other phages such as the typhoid Vi phages (Anderson & Felix, 1953) suggests that phenotypic variation and genetic mutation should also be considered as possible mechanisms of the origin of staphylococcal phages with altered host ranges or antigenic structure. Rippon (1952) described mutation of antigenic structure from group B to group F in the staphylococcal typing phage 42D. Ralston & Krueger (1954) produced evidence of phenotypic variation in host range of phage K (not a typing phage) dependent on the host used for its propagation.

During phage typing of staphylococci the application of a phage results, on occasion, in an area of confluent lysis covered with a thin veil of secondary growth which contains a few plaques larger in size than those of the phage originally applied. This appearance was noted frequently with phage 47C and, on isolation, phage from the large plaque was found to belong to serological group B, whereas 47C is group A (Rountree, 1949*b*). The origin of this group B phage was therefore investigated. During subsequent work, the use of a number of different hosts for the propagation of phage 47C and of the group B phage



revealed examples of changes in host range which appeared to be due to phenotypic variation. In addition, a virulent mutant of the group B phage was isolated. The typing patterns of various host staphylococci were altered by lysogenization with the two phages.

### METHODS

Phage stocks were prepared either in broth or on agar by methods described previously (Rountree, 1949*b*, 1952). Phage counts were made in quadruplicate by plating 0.01 ml. samples of the appropriate dilution on the surface of nutrient agar plates previously flooded with the appropriate propagating strain of staphylococcus.

The host range of the phage was examined in the first place by the method of Williams & Rippon (1952) using the propagating strains of staphylococci of Wilson & Atkinson (1945). More accurate determinations of host range were made by counting the number of plaques produced by a phage on all the strains sensitive to it. Strains of staphylococci were tested for lysogenicity by growing them in nutrient broth at 37° for 2–3 hr., centrifuging (angle centrifuge) and plating the supernatant fluids on appropriate indicator strains.

Phage neutralization tests with antisera prepared in rabbits were incubated at 37° for 90 min. before plating.

*Staphylococci.* Five staphylococcal strains were used to propagate phage 47C. *Staphylococcus* 1163 Pa was obtained from the Staphylococcal Reference Laboratory, Colindale, London, in 1946, and has been maintained since then on agar slopes and used as the propagating strain for phages 42B and 47C. *Staphylococcus* 1163 Col is another culture of strain 1163 obtained from Colindale in April 1953. These strains will be referred to as Pa and Col. *Staphylococcus* strains 7104, 7167 and 7296 were isolated from patients in this hospital. The phage-typing patterns of these strains are shown in Table 1. Since the lysogenic state of the strains was of some significance in the present work, the table includes data on their lysogenicity.

Table 1. *The phage-typing patterns and lysogenicity of strains of Staphylococcus aureus used to propagate phage 47C*

Strain	Phage pattern	Character of lysogenic phage present in strain	
		Serological type	Strains lysed by the phage
Pa	42B/47C/52	—	—
Col	42B/47C	B	6, 7, 31/44, 44A, 47, Pa, 7296
7104	29/42B/47C	B	31/44, 47
7167	29/42B/47C/52/52A	B	6, 7, 31/44, 42C, 42E, 44A, 47, 7296
7276	6/7/31B/42B/42E/44/47/47C/47D/52/52A/53/54/75	Not A, B, F or G	6, 47

*Phages.* Phage 47C was obtained from Colindale in 1946. The stock used as the starting material for the present work was made in broth in December 1952,

using strain Pa for propagation. Phage stocks are designated by the number or letter of that strain followed by that of the strain on which it was propagated. e.g. phage Col/Pa is a lysogenic phage from propagating strain Col grown in propagating strain Pa. When strains have been lysogenized the presence of the lysogenic phage is indicated in parentheses after the strain number or letter, e.g. strain Pa (47C) carries phage 47C.

*Origin of the group B phage present in stocks of 47C/Pa*

Phage 47C/Pa produced equal numbers of plaques when plated on propagating strains Pa, Col, 7104 or 7167 and slightly fewer plaques on strain 7296. The group B phage present in the stock phage could be detected only on strains Pa and 7296, on which it appeared as large plaques in the secondary growth covering the area of confluent lysis produced by undiluted and  $10^{-1}$  dilutions of phage 47C. Strain Pa (47C) could also be used to count the B phage.

Seven stocks of phage 47C/Pa prepared either in broth or on agar using customary inocula of 1/20 or 1/100 dilutions of the stock phage contained B phage in ratios of one B particle to  $10^5$  A particles. Such ratios suggested the possibility of a mutation of A to B.

Using single plaques from plates inoculated with a  $10^{-5}$  dilution of phage 47C/Pa 28 stocks were prepared. The use of this limiting dilution should ensure that these stocks originated from single particles of A phage. The contents of single plaques when picked into 0.1–0.2 ml. broth and then spread on plates flooded with propagating strain Pa produced confluent or semi-confluent lysis. Preparations from these plates contained  $10^8$ – $10^{10}$  particles A phage/ml. B phage was detected in only one preparation which contained  $6 \times 10^9$  A particles/ml. and  $1.5 \times 10^6$  B particles/ml. This relatively high titre of B suggested that the stock had been made from a mixed plaque resulting from the chance juxtaposition of the two types of particle during the original dilutions and plating of the phage. The absence of phage B from the other 27 indicated that it was unlikely that its occurrence was due to a mutation of antigenic structure.

Propagating strains Pa and Col were examined for lysogenicity. While strain Col released into broth large amounts of a B phage which lysed strain Pa, no lysogenic phage was detected in strain Pa, in spite of repeated testing. When phage from a single plaque of phage 47C/Pa was propagated on strain Col the resultant preparation contained a high titre of A and a small amount of B. Propagating strain Pa could be lysogenized with the B phage from strain Col and was then resistant to that phage. When typed propagating strain Pa(Col) gave the same typing pattern as strain Col, its sensitivity to phage 52 being abolished.

A further culture of staphylococcus 1163, obtained from Dr R. Wahl (Institut Pasteur, Paris) who had received it from Colindale in 1948, was lysogenic for a phage identical with that present in propagating strain Col. Furthermore, staphylococcus 1163 was known to have been lysogenic at Colindale in 1948 (Rountree, 1949*b*).

It was concluded that the presence of the B phage in stocks of phage 47C/Pa could be explained on the assumption that it was lysogenic phage from propagating strain Col. As some time after its arrival in our laboratory our strain of staphylococcus 1163 had lost its lysogenicity and its lysogenic phage was then perpetuated in stocks of phage 47C as a lytic phage. All stocks of phage 47C made in propagating strain Col and in strain Pa(Col) contain small amounts of the B phage as a contaminant, but pure stocks of 47C can be made in propagating strain Pa.

*Characters of the phages 47C/Pa and Col/Pa*

When propagated in staphylococcus strain Pa, the two phages displayed differences in plaque size, adsorption constants in broth (Rountree, 1952), requirements of divalent cations for penetration and growth (Rountree, 1955), length of the minimum latent period and average burst size (Table 2). Their ability to show plaques on a number of indicator strains of staphylococci is given in Table 3 and expressed as: (a) numbers of plaques/ml.; (b) the ratio of plaques/ml. on propagating strain Pa to plaques/ml. on the indicator strain. The values of (b) to the nearest round number provide a convenient means of

Table 2. *Characters of staphylococcal phages 47C/Pa and Col/Pa*

	Phage 47C/Pa	Phage Col/Pa
Serological group	A	B
Mean plaque size (mm.)	0.25	0.8
Adsorption constant (K)	$20 \times 10^{-11}$ cm. <sup>3</sup> /min. <sup>-1</sup>	$53 \times 10^{-11}$ cm. <sup>3</sup> /min. <sup>-1</sup>
Minimum latent period (min.)	60-65	40
Activity of divalent cations in adsorption and penetration	$\left\{ \begin{array}{l} \text{Ca}++* \\ \text{Mg}+\pm \\ \text{Sr}+ \end{array} \right.$	$\left\{ \begin{array}{l} \text{Ca}++ \\ \text{Mg}+ \\ \text{Sr}- \end{array} \right.$
Average burst size	16	90
Concentration CaCl <sub>2</sub> /ml. for maximum average burst size (μg)	100	340

\* ++ = cation active, + ± and + = cation partially active compared with Ca, - = no activity.

Table 3. *Host ranges of phages 47C/Pa and Col/Pa*

Host ranges expressed as (a) plaques/ml. on strain Pa and on a series of indicator strains, and (b) ratio of plaques/ml. on strain Pa to plaques/ml. on the particular indicator (IS).

Indicator strain of staphylococcus	Phage 47C/Pa		Phage Col/Pa	
	Plaques/ml. (a)	Ratio: Pa/IS (b)	Plaques/ml. (a)	Ratio (b)
Pa	$1.3 \times 10^9$	1	$2.4 \times 10^8$	1
Col	$1.1 \times 10^9$	1	Nil	.
7104	$1.6 \times 10^9$	1	$c. 7 \times 10^6$	c. 30
7167	$1.2 \times 10^9$	1	$c. 7 \times 10^6$	c. 30
7296	$4 \times 10^8$	3	$8.5 \times 10^7$	3
6	$6 \times 10^7$	20	$1.1 \times 10^6$	220
7	$2.6 \times 10^8$	500	$5.9 \times 10^5$	400
29A	Nil		$1 \times 10^4$	24,000
31/44	$3 \times 10^5$	4000	$6 \times 10^5$	400
44A	$3 \times 10^5$	4000	$2.5 \times 10^5$	1,000
47	$1.8 \times 10^7$	70	$1.9 \times 10^6$	130



comparison of host ranges. The phages were tested with the remainder of Wilson and Atkinson's propagating strains of staphylococci but lysed none of them.

Only minor differences were found in the host ranges of the two phages. Propagating strain Col was resistant to phage Col/Pa, and strains 7104 and 7167 were partly resistant, displaying only minute and fuzzy plaques which were impossible to count accurately, whereas phage 47C/Pa showed plaques equally well on all three strains of staphylococci. Phage Col/Pa produced a small number of plaques on propagating strain 29A while no preparations of phage 47C did so.

The shorter latent period and larger burst size of phage Col/Pa compared with phage 47C/Pa (Table 2) might have been expected to give it a selective advantage when the two phages were growing together in the original stocks of 47C/Pa. That phage Col/Pa did not replace 47C/Pa may have been due to the high calcium requirement of the former.

#### *The effect of lysogenization on typing patterns*

The staphylococcal strains Col, Pa, 7104, 7167 and 7296 differ in their typing patterns (Table 1). The lysogenic phages carried by strains Col, 7104, 7167 and 7296 differ in host range, and, in the case of 7269, in serology. Since the difference in typing pattern between propagating strains Col and Pa might have been correlated with a lack of lysogenicity of strain Pa, the effect of further lysogenization of the five strains of staphylococci was studied. The strains to be lysogenized were grown in broth or on agar, together with the appropriate undiluted phage for 24–48 hr. at 37°. The resulting resistant growth was subcultured on agar plates and, after incubation, single colonies were picked into broth. These cultures were then tested for changes in phage-typing pattern, for resistance to the phage used for lysogenization and for the presence of this phage in the lysogenic state. In the latter test, the homologous non-lysogenized strain was used as indicator strain in order to avoid confusion of the newly introduced phage with lysogenic phage or phages already present in the strain.

Propagating strain Pa was lysogenized with phages 47C and Col separately and by both phages one after the other; the order in which the two phages were introduced made no difference. Propagating strains Col, 7104, 7167 and 7296 were lysogenized with phage 47C. Propagating strains 7104 (47C), 7167, 7167 (47C) and 7296 were lysogenized with phage Col. Attempts to lysogenize propagating strain 7296 (47C) with phage Col were unsuccessful. All the lysogenized strains were resistant to the phages introduced into them. The effect of lysogenization on their typing patterns is shown in Table 4.

In propagating strains Pa, Col, 7104 and 7167 lysogenization with phage 47C conferred immunity only to this phage. In propagating strain 7296 (a strain sensitive to an extended range of phages) lysogenization with phage 47C resulted in resistance to phages 6, 7, 42E, 47, 47D and 54 as well as to phage 47C, all of which are serologically A.

The introduction of phage Col into propagating strain Pa resulted in immunity to phage 52, into strain 7104, in immunity to phage 29 and into



strain 7167, in immunity to phages 29, 52 and 52A. In propagating strain 7296, sensitivity to phages 31B, 44, 52 and 52A was abolished. It should be noted that phage Col does not lyse the propagating strains of any of these phages, all of which are serologically B. The doubly lysogenic staphylococcal strains Pa (47C, Col), 7104 (47C, Col) and 7167 (47C, Col) reacted only with phage 42B and were therefore identical in typing pattern with PS Col (47C).

Table 4. *Effect of lysogenization with phages 47C and Col on the typing pattern of strains of Staphylococcus aureus*

Strain designation*	Phage typing pattern	Phage reactions abolished by lysogenization
Col (47C)*	42B	47C
Pa (47C)	42B/52	47C
Pa (Col)	42B/47C	52
Pa (47C, Col)	42B	47C/52
Pa (Col, 47C)	42B	47C/52
7104 (47C)	29/42B	47C
7104 (47C, Col)	42B	29/47C
7167 (47C)	29/42B/52/52A	47C
7167 (Col)	42B/47C	29/52/52A
7167 (47C, Col)	42B	29/47C/52/52A
7296 (47C)	31B/42B/44/52/52A/53/75	6/7/42E/47/47C/47D/54
7296 (Col)	6/7/42B/42E/47/47C/47D/53/54/75	31B/44/52/52A

\* The lysogenic phages introduced into the strains are indicated in parentheses.

Lwoff's hypothesis (1953) that the presence of a particular prophage at a specific site in the bacterial cell confers immunity to the homologous infecting phage can explain these results, if it be assumed that the receptor sites for antigenically similar staphylococcal phages are either identical or contiguous, and that the occupation of these sites prevents the replication of related infecting phages. Antigenically and in host range the phages to which immunity is conferred by the prophages of Col and 47C fall into two distinct groups. Since both these prophages could be introduced into the five staphylococcal strains used, it can be assumed that each occupies a different site in the lysogenized cells. The phage carried by strains 7296 is antigenically different from both 47C and Col and its presence in propagating strains 7296 did not prevent lysogenization with either of these two phages; it can be assumed therefore that it occupies a different site in propagating strain 7296. That the sites involved in lysogenization with and attack by antigenically similar phages may be contiguous rather than identical is suggested by the findings with propagating strains 7104 and 7167. These two strains already carry B prophages, apparently not identical in host range with that of propagating strain Col, and their partial resistance to phage Col may be due to the presence of these prophages. Nevertheless, they can be lysogenized with phage Col.

#### *Phenotypic modification of phages 47C and Col*

*Modification of phage 47C.* Stocks of phage 47C prepared on agar with propagating strains Col, Pa, 7104, 7167 and 7296 as host organisms showed differences in their host ranges when expressed as ratios of plaques on the

strain used for propagation to plaques on a series of indicator strains. Table 4 shows that the host range of phage 47C/Col did not differ essentially from that of phage 47C/Pa. Phages 47C/7104 and 47C/7167 resembled each other but differed from the others. Phage 47C/7296 was unlike the others. The chief differences were: (1) the loss of ability to form plaques on propagating strains Col and Pa after propagation of the phage in strains 7104, 7167 and 7296; (2) the acquisition of ability to form plaques on propagating strains 6 and 47 after propagation in strain 7296.

Table 5. *Change in host range with change in propagating strain of phage 47C*

Titre/ml. on propagating strain	Bacteriophages				
	47C/Pa	47C/Col	47C/7104	47C/7167	47C/7296
	$1.3 \times 10^9$	$5 \times 10^8$	$1.3 \times 10^9$	$1.6 \times 10^9$	$3.6 \times 10^9$
Ratio: Titre on propagating strain to titre on indicator strain					
Indicator strain Pa	1	1	46,000	48,000	560,000
Col	1	1	46,000	4,000	56,000
7104	1	1	1	1	50
7167	1	1	1	1	150
7296	3	3	200	80	1
6	20	50	130	90	1
7	500	1,000	900	2,100	3,500
31/44	4,000	2,000	1,430	2,600	3,000
44A	4,000	32,000	4,100	4,800	1,300
47	70	50	110	120	1

When phage 47C/7104 was re-propagated in strain Pa, the phage obtained was identical with 47C/Pa and 47C/Col. Since the original stock of 47C/Pa had given the same titre whether plated on propagating strains Pa, Col, 7104 or 7167, it was considered that the change after growth in propagating strains 7104 and 7167 probably represented host-controlled modification of the particles rather than selection of mutants.

The change in phage 47C/7104 was examined on agar plates. After incubation of plates inoculated with propagating strain 7104 and a  $10^{-1}$  dilution of phage 47C/Pa, the entire contents of 10 plaques were each picked into 0.5 ml. broth, by using a Pasteur pipette drawn out to a capillary. After thorough mixing, samples were replated on propagating strains 7104 and Col. Table 5 shows the number of phage particles/plaque on the two strains and the ratios between the counts. There was a loss of ability to form plaques on propagating strain Col which varied from plaque to plaque, the ratios ranging from 12:1 to 533:1. The plaques on propagating strain Col were (with a few exceptions to be discussed later) minute and difficult to see without a hand-lens. These ratios were lower than that found in the original propagation of phage 47C/7104, viz. 46,000:1.

The changes in phage 47C/7104 might be explained on the assumption that there was a gradual loss of ability to attack staphylococcal strains Col and Pa after numerous cycles of replication of phage 47C in the cells of other hosts, such as strains 7104 and 7167. Alternatively, one might postulate that, in the

successive passages of the phage from cell to cell of strain 7104 which will occur when a high titre phage is prepared by confluent lysis on agar plates, selection of 'fitter' (in the sense of their ability to absorb to, or replicate in, cells of strain 7104) phage particles takes place. In either case, the character of the host cells would direct the change. The differences in ratios between the various plaques and the large stock of phage 47C/7104 would depend on variation in the numbers of cycles of replication of the phage in the various preparations.

Table 6. *Phage titre of single plaques of phage 47C/7104 when plated on strains 7104 and Col.*

Plaque no.	Particles/plaque		Ratio a/b
	On strain 7104 (a)	On strain Col (b)	
1	$5.6 \times 10^5$	$2.5 \times 10^3$	220
2	$2.1 \times 10^6$	$7.5 \times 10^3$	280
3	$1.5 \times 10^6$	$3 \times 10^3$	533
4	$8.3 \times 10^5$	$5 \times 10^3$	166
5	$3.6 \times 10^6$	$9.5 \times 10^3$	377
6	$2.5 \times 10^6$	$1.3 \times 10^4$	192
7	$6.4 \times 10^5$	$2.5 \times 10^3$	256
8	$5.4 \times 10^5$	$3 \times 10^3$	180
9	$1.3 \times 10^6$	$2.8 \times 10^4$	46
10	$3.9 \times 10^5$	$3.2 \times 10^4$	112

That the character of the host cell has an important effect on the nature of the phage propagated in it was further demonstrated when the lysogenic phage from propagating strains 7104 (47C), 7167 (47C) and 7296 (47C) was examined. The phage 47C which had been introduced into these strains was present in broth cultures at titres of  $10^5$ – $10^6$  particles/ml. and showed plaques on propagating strains 7104, 7167 or 7296 but not at all on propagating strains Pa or Col, having lost completely the ability to replicate in its former hosts.

By growing the phage in broth culture, the change in phage 47C/7296 in which ability was acquired to show plaques with equal efficiency on propagating strains 6 and 47 was found to occur in one cycle of replication. The cells of propagating strain 7296 were infected with phage 47C/Pa (pure A); the yield after one step and after complete clearing of the culture is given in Table 7. After one step, equal phage counts were obtained on propagating strains 7296, Pa, Col, 7104, 7167, 6 and 47. After several steps, the ability to form plaques on propagating strains Pa, Col, 7104 and 7167 was diminished.

*Variation in the lysogenic phage from strain Col.* When propagating strain Col was grown with aeration in glucose veal broth, large amounts of phage were released into the medium. This lysogenic phage was harvested after 2 hr. incubation and titrated on the series of indicator strains of staphylococci which were known to be sensitive to it. The titres (Table 8) varied from strain to strain, propagating strain Pa giving the highest count and propagating strains 7104, 7167, 7, and 44A giving similar counts. This lysogenic phage was then propagated from single plaques in propagating strains Pa, 7296, 6, 31/44, 44A,

and 47. The host range ratios of phages Col/Pa and Col/7296 (Table 8) and the host ranges of the other four preparations (expressed in Table 9 by Williams & Rippon's method) all indicate changes in the lytic spectra of the phage according to the hosts used for propagation. All phage preparations were serologically B. Phage Col/7296 behaved like phage 47C/7296 in that it showed plaques

Table 7. *Change in host range of phage 47C/Pa after one and after several cycles of replication in strain 7296*

Indicator strain (IS)	Phage 47C/Pa		One cycle in strain 7296		Several cycles in strain 7296	
	Plaques/ml.	Ratio: Pa/IS	Plaques/ml.	Ratio: 7296/IS	Plaques/ml.	Ratio: 7296/IS
7296	$4 \times 10^8$	3	$7.5 \times 10^7$	1	$1.4 \times 10^9$	1
Pa	$1.3 \times 10^9$	1	$1 \times 10^8$	1	$8 \times 10^6$	175
Col	$1.1 \times 10^9$	1	$8 \times 10^7$	1	$8 \times 10^6$	175
7104	$1.6 \times 10^9$	1	$6 \times 10^7$	1	$1.2 \times 10^8$	12
7167	$1.2 \times 10^9$	1	$7 \times 10^7$	1	$6 \times 10^7$	23
6	$6 \times 10^7$	20	$6.5 \times 10^7$	1	$7.5 \times 10^8$	1.8
7	$2.6 \times 10^6$	500	$1.3 \times 10^4$	6000	$6 \times 10^6$	500
31/44	$3 \times 10^5$	4000	$5.5 \times 10^4$	1500	$7 \times 10^4$	20,000
44A	$3 \times 10^5$	4000	$5 \times 10^4$	1500	$4.5 \times 10^4$	30,000
47	$1.8 \times 10^7$	70	$7 \times 10^7$	1	$1.6 \times 10^9$	1

Table 8. *Modifications of the host range of lysogenic phage from strain Col following its propagation in strains Pa and 7296*

Indicator strain	Phage Col on release from strain Col		Phage Col/Pa		Phage Col/7296	
	Titre/ml.	Ratio: Pa/IS	Titre/ml.	Ratio: Pa/IS	Titre/ml.	Ratio: 7296/IS
Pa	$8 \times 10^6$		$2.4 \times 10^8$		$1.9 \times 10^9$	28
7104	$2 \times 10^5$	40	<i>c.</i> $7 \times 10^8$ *	<i>c.</i> 34*	<i>c.</i> $5 \times 10^8$ *	<i>c.</i> 100*
7167	$2 \times 10^5$	40	<i>c.</i> $7 \times 10^8$ *	<i>c.</i> 34*	<i>c.</i> $5 \times 10^8$ *	<i>c.</i> 100*
7296	$2.5 \times 10^6$	3	$8.5 \times 10^7$	2.8	$5.5 \times 10^{10}$	
6	$9 \times 10^4$	90	$1.1 \times 10^8$	218	$2.4 \times 10^{10}$	2.3
7	$1.7 \times 10^5$	47	$5.9 \times 10^5$	400	$5.2 \times 10^8$	100
29A	$1.5 \times 10^2$	50,000	$1 \times 10^4$	29,000	$5 \times 10^6$	10,000
31/44	$4 \times 10^5$	20	$6 \times 10^5$	400	$5 \times 10^7$	1,000
44A	$1.8 \times 10^5$	44	$2.5 \times 10^5$	960	$1.6 \times 10^8$	340
47	$6.5 \times 10^6$	12	$1.9 \times 10^6$	130	$4.4 \times 10^{10}$	1.3

\* Approximate counts, strains being partly resistant.

more efficiently on propagating strains 6 and 47 than did the original phage. The results indicated that phages 47C and Col were both influenced in their host ranges by the cells in which they had been replicated. In cases where the phage could be transferred back to its original host it regained its previous lytic spectrum, indicating that the changes were phenotypic ones. In certain hosts, prolonged contact as prophage in the new host abolished completely the ability of the phage to attack former hosts.



Table 9. *Modification of the host range of lysogenic phage Col, following its propagation in various hosts*

Indicator strain	Phage			
	Col/6	Col/31/44	Col/44A	Col/47
Pa	++++	++++	++++	++++
7104	—	—	—	—
7167	—	—	—	—
7296	++++	++	++++	++++
6	++++	+	++	+
7	++	±	++	±
31/44	++	++++	++	++
44A	+	—	++++	—
47	++++	++++	+	++++

++++ = confluent lysis. ++ = numerous plaques. + = 10–20 plaques.  
 ± = < 1–9 plaques. — = no plaques.

#### *Induction of a virulent mutant of phage Col*

As already described, when phages 47C/7104 and 47C/7167 were plated on propagating strain Col they showed poor efficiency in showing plaques, and these were small. However, some plaques were larger and clearer than others; such plaques were not found on propagating strain Pa. When the contents of these plaques were examined serologically, they were found to consist of particles of serological group B. Three possible origins of this B phage were considered: (1) it was lysogenic phage derived from propagating strains 7104 or 7167; (2) it was a recombinant of phage 47C/7104 or 47C/7167 and the prophage of Col; (3) it was a virulent mutant of Col prophage. If (1) were the correct explanation, phage capable of lysing propagating strain Col should be detectable in the supernatant fluids of propagating strains 7104 and 7167. Furthermore, stocks of phages 47C/7104 and 47C/7167 should contain the B particles and treatment with anti-B serum before the phages were applied to plates of propagating strain Col should prevent the appearance of the B plaques. No evidence of lysogenic phage capable of lysing propagating strain Col was found on repeated examination of the supernatant fluids of cultures of propagating strains 7104 and 7167.

Quantities (0.1 ml.) of suspensions of phages 47C/7104 and 47C/7167 were mixed with 0.1 ml. quantities of  $10^{-2}$  dilutions of high titre anti-A and anti-B sera and with both sera. Control tubes containing only phage and broth were included. After incubation at 37° for 90 min., 0.01 ml. samples were spread on plates of propagating strains Pa and Col. After incubation, no large plaques were found on the Pa plates. On propagating strain Col large and small plaques were present on plates which had been inoculated with the phage treated with anti-B serum and with the untreated phage; no plaques of any kind were present on the plates inoculated with phage pretreated with anti-A serum. The serology of a number of the large and small plaques was examined. The large plaques were B; the small were A; no plaques containing both types of particles were found. It was concluded that the B phage was not present in the stocks of phages 47C/7104 and 47C/7167, but that it had arisen following

contact of these phages with the Col cells. The fact that no mixed plaques were found ruled out the possibility of a recombination of phage 47C with the B prophage of strain Col. The most reasonable explanation of these findings was that the B phage had arisen by induction of a virulent mutant of Col prophage, analogous to the virulent mutant of  $\lambda$  prophage of *Escherichia coli*, K12 which is induced by ultraviolet irradiated  $\lambda$  phage (Jacob & Wollman, 1953). This explanation was supported by the findings that B phage was not produced on the non-lysogenic Pa cells and that when Pa (Col) cells were used for plating the B phage was obtained.

Three other phages, Col/7296, 7104/31 and 7104/47, showed what was probably the same inducing effect, the phage appearing on propagating strains Col and Pa (Col) but not on strain Pa. Since they were antigenically similar to the mutant serological prevention of induction could not be applied to them. However, evidence was not found that either of the propagating strains 7296, 31/44 or 47 carried phage capable of infecting strains Col or Pa.

The host ranges of four preparations of the mutant phage of different origins made in propagating strain Col were identical (Table 10); they differed from those of phage Col. In particular, none produced plaques on propagating strain 7104, although  $10^{-1}$  dilutions gave zones of inhibition such as described by Williams & Rippon (1952) with some typing phages. The mutant phage often gave higher plaque counts on propagating strain Pa than on strain Col, suggesting that abortive infections occurred in cells containing the prophage which had been the origin of the mutant.

Table 10. *Phage counts/ml. on various indicator strains of four separate preparations of the virulent mutant of prophage Col*

Indicator strain	Phage preparation			
	47C/7104/Col	Col/7296/Col	7104/31/Col	7104/47/Col
Col	$3.3 \times 10^9$	$1.8 \times 10^9$	$7.9 \times 10^9$	$6.4 \times 10^9$
Pa	$4.6 \times 10^9$	$1.8 \times 10^9$	$1 \times 10^{10}$	$8.5 \times 10^9$
7104	Inhibition	Inhibition	Inhibition	Inhibition
7167	$9 \times 10^6$	$2.7 \times 10^7$	$4.5 \times 10^7$	$3 \times 10^7$
7296	$2.3 \times 10^8$	$1.4 \times 10^6$	$3 \times 10^7$	$2.4 \times 10^7$
6	$1.1 \times 10^6$	$3 \times 10^5$	$4.5 \times 10^6$	$3 \times 10^5$
7	$1 \times 10^7$	$1.4 \times 10^6$	$3 \times 10^6$	$2.5 \times 10^6$
29A	$1.8 \times 10^5$	$1.5 \times 10^5$	$4 \times 10^5$	$3 \times 10^5$
31	$9 \times 10^6$	$2.9 \times 10^6$	$3 \times 10^7$	$1.7 \times 10^7$
44A	$7.5 \times 10^6$	$1.8 \times 10^6$	$5 \times 10^6$	$5 \times 10^6$
47	$1.5 \times 10^7$	$1.3 \times 10^6$	$2 \times 10^7$	$1.9 \times 10^7$

Conditions under which the induced phage occurred appear to involve the application to Col cultures of a large number of phage particles which lack ability to be replicated in Col cells. The mutant was not detected when phages 47C/Pa or 47C/7296 were plated on Col cells. The ratio of inducing phage to induced mutant is shown in Table 11. The case of phage Col/7296 was of particular interest since it indicated that, after replication of phage Col in propagating strain 7296, a small proportion of the particles were able to act as inducers of a mutant of the prophage from which they originally arose.

Table 11. *Ratio of inducing phage inoculated on strain Col to plaques of the mutant formed*

Inducing phage		No. of phage particles inoculated (a)	No. of B plaques formed (b)	Ratio a/b
Designation	Serology			
Col/7296	B	$5.5 \times 10^8$	4	$1.4 \times 10^8$
47 C/7104	A	$6 \times 10^6$	15	$4 \times 10^5$
47 C/7167	A	$8 \times 10^6$	13	$5 \times 10^5$
7104/31	B	$6 \times 10^6$	140	$4 \times 10^4$
7104/47	B	$1.9 \times 10^6$	500	$4 \times 10^3$

## DISCUSSION

It is generally assumed that stocks (clones) of bacteriophages made from single plaques consist of genetically homogeneous populations of particles. However, mutations of host range and plaque morphology occur in many phages, e.g. the coli T phages (Luria, 1945) and phage  $\lambda$  (Jacob & Wollman, 1953), and phenotypic variation involving altered host range and dependent on the host, has been described in the coli-dysentery phages  $\lambda$  and P2 (Bertani & Weigle, 1953), in coli phage T2 (Luria & Human, 1952) and in Vi typing phages (Anderson & Fraser, 1955). In the staphylococcal phages, interpretation of changes arising in phage clones is complicated by the almost universal lysogenicity of the cocci. The amount of lysogenic phage released by the host cells varies from strain to strain and many presumably pure phages may be contaminated in this way. Mutation of prophage to virulence during the synthesis of another phage or recombination of prophage with another phage during a lytic cycle are also possible events which might add to the complexity of phage stocks.

The present results suggest that the changes in host range of phages 47C and Col were due to modification of the particles by their host cells rather than to selection of mutants in the original phage stocks. In the phages grown in propagating strain 7296 which acquired ability to infect certain hosts the change occurred in one cycle of replication and can therefore be regarded as phenotypic. In those phages in which alterations in host range involved loss of ability to infect, there was evidence that the duration of contact of the particles with their new host determined the extent of this loss. In particular, long contact as the result of lysogenization caused profound alterations in the host range of the phage. In such cases, the selection of genotypic mutants during the propagation of the phage in its new host cannot be excluded with certainty.

One question of interest is whether prophage already present in a particular coccus may influence the character of another phage growing in the same organism. For example, cells of propagating strain 7296 carry a phage which lyses strongly propagating strains 6 and 47, and phages 47C and Col both acquired similar lytic ability after passage through propagating strain 7296, suggesting some interaction between the prophage and the lytic phage. On the other hand, propagating strains 7104 and 7167 both carry phages which lyse propagating strains 31/44 and 47, but after phage 47C had been grown in these



two strains it did not acquire any increased ability to attack strains 31/44 or 47. Perhaps the association found in propagating strain 7296 was fortuitous but there is the possibility that the prophage must be situated at a particular locus in the host cell before it can influence the character of other phages.

Williams Smith (1948) and Lowbury & Hood (1953) showed that lysogenization of staphylococci altered their sensitivity to the typing phages. The present results confirm their observations and also indicate that the immunity conferred on a staphylococcal strain by a particular prophage may be either quite narrow and relate only to the lysogenizing phage or may extend to a number of phages. Which of these effects occur depends on the original typing pattern of the staphylococcus and this may be due to some intrinsic character of the strain as well as to its original lysogenic state.

Cross-immunity was not observed between phages of different antigenic structure but was found between phages which on serological and other grounds (usually host range) had been considered closely related. Many of the typing phages which differ only in their host ranges may, in fact, be phenotypic variants. The occurrence of reciprocal cross-immunity following lysogenization should therefore provide useful evidence of the identity of phages suspected of being closely related or identical.

The changes produced in phages 47C and Col by their transfer to different hosts have some application to the practice of phage typing of staphylococci. They illustrate the inadvisability of using any other than the standard strains for the propagation of phage stocks. The practice of testing with undiluted phages strains not lysed at the routine test dilution and of assuming, when lysis occurs, that it is due to the particular phage or phages applied, may need further consideration in the light of the finding that a virulent mutant of Col prophage could be induced by the application of a number of different phages. If such a phenomenon is of frequent occurrence, it would be unwise to assume that patterns obtained in typing by the application of high titre phages are due to the lysis of the host by these phages. Furthermore, some 'adapted' phages obtained by this method may be induced virulent mutants.

The apparent heterogeneity of the lysogenic phage released by Col cells poses a special problem. When serological differences are found between several lysogenic phages released by a particular staphylococcal strain, such as occur with phages belonging to group II (Rountree, 1949*b*), then multiple lysogenicity can be assumed. When, however, the differences are those of host range it may be incorrect to assume that distinct phages are present. Changes in host range occurred when the phage released in Col cultures was propagated in a variety of hosts. The available evidence suggests that these changes were phenotypic. The differences in efficiency in showing plaques revealed when the lysogenic phage in supernatant fluids of cultures of propagating strain Col was plated on the indicator strains may therefore be an expression of host differences rather than of differences in the released particles. In phage typing, differences in the degree of lysis caused by phages applied at their routine test dilutions are used as indications of host differences, but the exact significance and mechanism of reactions which vary from a few plaques to confluent lysis



with a particular phage are still undetermined. Differences in efficiency in showing plaques of phage Col may be of similar nature. Further work is required to decide whether these differences are due to inhomogeneity of phage particles or to differences in the hosts. It seems likely, however, that the phenomena are of the same character as those in coli-dysentery phages discussed by Weigle & Bertani (1953) who concluded that they arise from differences in the hosts.

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## The Essential Metabolites of a Strain of *Paramecium aurelia* (Stock 47.8) and a Comparison of the Growth Rate of Different Strains of *Paramecium aurelia* in Axenic Culture

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**SUMMARY:** *Paramecium aurelia*, var. 4, stock 47.8 (sensitive) grown in an axenic medium required the following amino acids: DL-tyrosine, L-phenylalanine, L-tryptophan, DL-methionine, DL-threonine, L-leucine, DL-isoleucine, L-lysine, L-histidine, L-arginine, and DL-serine. DL-valine, L-proline and glycine were synthesized at a slow rate. Tyrosine, methionine and serine were required by stock 51.7 (sensitive). Other essential metabolites required by stock 47.8 (s) were thiamine, riboflavin, folic acid, nicotinic acid, pantothenic acid, and possibly pyridoxal. 2,6-diaminopurine inhibited growth at concentrations as low as 1-4  $\mu\text{g./ml.}$  The growth rates (expressed as final population density/ml./transfer) of various stocks in identical media are different. An apparent correlation was found to exist between the growth rates and the chromosome number of these stocks, those with the largest chromosome number attaining the highest population density.

*Paramecium aurelia* was successfully established in axenic culture by van Wagtendonk & Hackett (1949). The heat-sterilized medium was composed of equal parts of a 0.5% yeast autolysate (Basamin-Busch) and an autoclaved, 24 hr. culture of *Aerobacter aerogenes* in lettuce extract. Subsequent work led to the development of a medium consisting of salts, B-vitamins, a plant extract, a yeast extract, proteose-peptone and guanylic and cytidylic acids (van Wagtendonk, Miller & Conner, 1952). The use of this medium made it possible to show that folic acid, riboflavin, thiamine and a steroid were absolute growth requirements for stock 57.1 (s) (van Wagtendonk, Conner, Miller & Rao, 1953; Conner, van Wagtendonk & Miller, 1953).

Additional stocks of *Paramecium aurelia* have now been established in axenic culture and maintained through more than a hundred serial subcultures. The specific amino acid and vitamin requirements of stock 47.8 (s), and a comparison of the growth of various stocks and their requirements are reported here.

### METHODS

Five different stocks of *Paramecium aurelia*, variety 4, were examined in these experiments: 47.8 (s), 51.7 (s) and its opposite mating type 51.8 (s), 32.7 (s), and 29.7 (s). None of these stocks contained kappa. Axenic cultures of stock 51.7 (s) were available from earlier experiments, while the other stocks

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were freed from associated bacterial forms by treatment with penicillin after the method of van Wagtendonk & Hackett (1949). The organisms were cultured in 5 ml. of the test media in 15 × 150 mm. Pyrex tubes covered with metal caps. All experimental series were incubated at 27° for 5-day periods for each subculture. At the end of each 5-day incubation period an inoculum (0.1–0.3 ml.) was transferred to the next serial subculture. The total number of paramecia was determined by a direct count of measured samples from each tube and the counts from duplicate tubes were averaged to give the final population number. The growth obtained in the experimental tubes was expressed as a percentage of the control in each series, the control being arbitrarily taken as 100. All experiments were repeated at least once.

The method of preparation of the required unidentified factor(s) from yeast reported by van Wagtendonk *et al.* (1953) was modified. A 1/4 (w/v) suspension of Fleischmann's 'active dry' yeast was prepared by continuously stirring the yeast in distilled water at 50° for 2–3 hr. The suspension was autoclaved for 20 min. at 120° and centrifuged to collect a clear amber supernatant fluid. The supernatant fluid was again autoclaved in a section of cellulose dialysis tubing with one end left open to be tied off immediately after sterilization. This technique allowed for the exhaustive dialysis of large batches at room temperature without incurring contamination. The non-dialysable yeast fraction (NDF) was added aseptically to the basal medium given in Table 1. All the components of the medium may be autoclaved together in final concentration except the NDF and the B-vitamins which must be sterilized separately and added aseptically. All solutions were prepared with double distilled water; the pH value of the medium was 6.8–7.0.

Table 1. *Composition of basal medium for growth of Paramecium aurelia, var. 4, stock 47.8*

A non-dialysable yeast fraction (NDF) was added aseptically to this medium to give a complete medium. The B-vitamins were sterilized separately and added aseptically. The other components were autoclaved together in final concentration.

	Final concn. ( $\mu\text{g.}/\text{ml.}$ )		Final concn. ( $\mu\text{g.}/\text{ml.}$ )
MgSO <sub>4</sub> ·7H <sub>2</sub> O	40.0	Stigmasterol	0.5
Fe(NH <sub>4</sub> ) <sub>2</sub> (SO <sub>4</sub> ) <sub>2</sub> ·6H <sub>2</sub> O	10.0	Ca panthothenate	2.0
MnCl <sub>2</sub> ·4H <sub>2</sub> O	0.1	Nicotinamide	2.0
ZnCl <sub>2</sub>	0.02	Pyridoxal.HCl	2.0
CaCl <sub>2</sub> ·2H <sub>2</sub> O	20.0	Riboflavin	2.0
CuCl <sub>2</sub> ·2H <sub>2</sub> O	2.0	Folic acid	1.0
FeCl <sub>3</sub> ·6H <sub>2</sub> O	0.5	Thiamine	6.0
K <sub>2</sub> HPO <sub>4</sub>	570.0	Nitrogen source: (1 % proteose-peptone or amino acids as given in Table 2)	
KH <sub>2</sub> PO <sub>4</sub>	570.0		
Ethylenediamine tetraacetic acid	16.0		
Sodium acetate	570.0		
Sodium pyruvate	570.0		

Conner & van Wagtendonk (1955) showed that the steroid requirement of *Paramecium aurelia* stock 51.7, although fairly specific with regard to



molecular configuration, may be satisfied by any one of several steroids. A preliminary report on the need for steroids by stock 47.8 was published by Conner, (1955), and a steroid requirement for *P. multimicronucleatum* has been shown by Johnson & Miller (to be published). Thus, stigmasterol was included in all the media used in these experiments.

## RESULTS

### *The requirement for a non-dialysable factor (NDF) from yeast*

Studies are in progress to identify the unknown factor(s) present in the essential fraction from yeast. This component is an absolute requirement for all stocks of *Paramecium aurelia* thus far tested. Although the active yeast fraction used in these experiments remained quite complex, sufficient purification was obtained in the dialysis step alone to allow its use in the determination of several other essential metabolites. Upon dialysis of the initial aqueous extract from the yeast approximately 75 % of the total solids were removed, and a tenfold purification of the activity on a weight basis was effected. The average weight of several NDF preparations was 12.5 mg./ml. The response of stocks 47.8 and 51.7 to different concentrations of the NDF, in the complete medium with amino acids is given in Fig. 1. Concentrations below 0.5 mg./ml. would not support continued growth of the organisms. The non-dialysable yeast fraction gave a very faint ninhydrin reaction, a positive biuret reaction and a positive Benedict's test for reducing sugar after strong acid hydrolysis.

### *Nitrogen requirements*

As shown in Table 3 the nitrogen requirements of *Paramecium aurelia* can be met by proteose-peptone. This material is satisfactory for routine growth and in its presence the requirements for stigmasterol, folic acid, riboflavin, thiamine and the NDF can be shown. However, it became desirable to replace proteose-peptone with known compounds in order to determine whether it was masking additional vitamin requirements, and to allow for a more specific analysis of the nitrogen requirements.

An acid hydrolysis of proteose-peptone was carried out by refluxing it for several hours with hydrochloric acid. The acid was removed by vacuum distillation and with the aid of Amberlite IR-45 (OH), whereupon the hydrolysate was resolved on a two-dimensional paper chromatogram. The following amino acids were identified: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine. The presence of a small amount of cystine was also indicated.

Several preliminary experiments with stock 47.8 to test whether proteose-peptone could be replaced by its acid hydrolysate or by a mixture of the amino acids identified in the chromatogram indicated that either would permit growth provided tryptophan was also added. Different concentrations of the complete amino acid mixture and of some of the individual amino acids were tested to determine their approximate optimum concentrations. Table 2 lists the



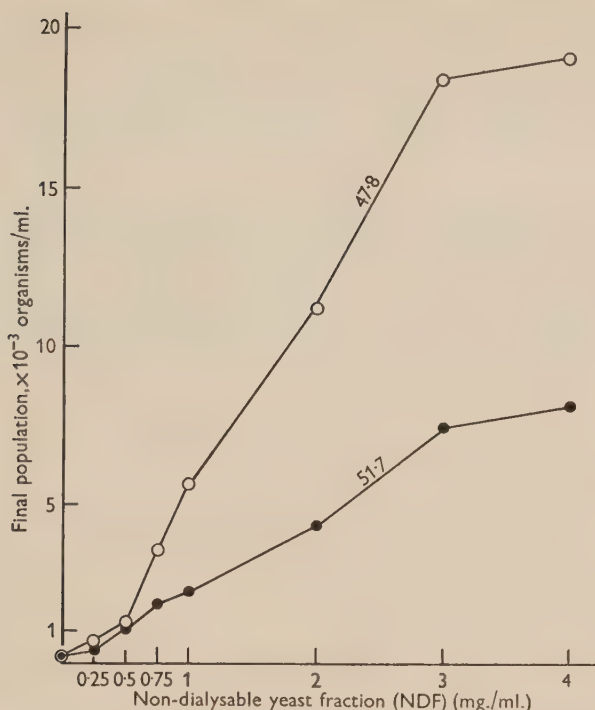


Fig. 1. The response of two stocks of *Paramecium aurelia* to varying concentrations of the non-dialysable yeast fraction (NDF) in the complete basal medium with amino acids. Concentrations of the NDF below 0.5 mg./ml. would not support continued growth of the organisms. ○, stock 47.8; ●, stock 51.7. The final population was determined 5 days after inoculation.

Table 2. *Mixture of amino acids used to replace proteose-peptone in the basal medium for the growth of Paramecium aurelia, var. 4, stock 47.8*

	Final concn. ( $\mu$ g./ml.)		Final concn. ( $\mu$ g./ml.)
L-Tryptophan	50	DL-Serine	200
DL-Methionine	150	L-Phenylalanine	75
DL-Threonine	150	L(-)-Tyrosine	50
DL-Isoleucine	150	L-Proline	50
L-Leucine	150	L-Alanine	25
L-Lysine	125	L-Aspartic acid	50
L-Histidine	50	L(+)-Glutamic acid	75
L-Arginine	100	Glycine	25
DL-Valine	75	L(-)-Hydroxyproline	100
		L-Cystine	50

concentrations which supported the most favourable growth. These concentrations were used throughout the experiments described below.

The results of experiments with organisms of stock 47.8 to compare the growth obtained with proteose-peptone, its hydrolysate, and amino acids as the nitrogen source are given in Table 3. These results clearly demonstrate that: (1) free amino acids were utilized and served as the major nitrogen source;

(2) the absence of specific single amino acids (tryptophan and tyrosine in this initial series) prevented or markedly limited continued growth; (3) the presence of one or more of the normally non-essential amino acids (alanine, aspartic acid, glutamic acid, glycine and proline) markedly stimulated growth. The absence of cystine or hydroxyproline did not impair growth.

Table 3. *A comparison of the growth of Paramecium aurelia, var. 4, stock 47.8 in media containing different nitrogen sources*

The nitrogen sources were tested in the complete basal medium using the NDF at a concentration of 2.5 mg./ml. A, 1% proteose peptone; B, an acid hydrolysate of proteose peptone (PP-H); C, PP-H plus tryptophan; D, a mixture of 18 amino acids contained in the PP-H plus tryptophan, as given in Table 2, (AA); E, AA minus hydroxyproline and cystine; F, AA minus tyrosine; G, AA minus alanine, proline, glycine, aspartic acid and glutamic acid.

Medium	Number of serial subcultures								
	1	2	3	4	5	6	7	8	9
	Percentage organisms/unit volume of medium as compared with proteose peptone taken as 100 %								
A	100	100	100	100	100	100	100	100	100
B	34	37	20	10	0	—	—	—	—
C	32	32	55	67	38	57	61	—	—
D	44	42	67	58	40	52	76	82	85
E	42	41	69	50	51	59	58	80	80
F	26	25	38	23	16	9	6	0	—
G	30	35	31	26	23	35	43	44	44

Using the single omission technique, extended series were designed to determine the need by organisms of stock 47 and 51 for each of the following amino acids: serine, methionine, phenylalanine, tyrosine, tryptophan, threonine, isoleucine, leucine, lysine, histidine, valine, and arginine (Table 4). Both stocks require methionine, serine and tyrosine; stock 47.8 also showed the need for histidine.

Two to four mg. NDF/ml. were used in the medium for these initial experiments. The results indicated that either the NDF, at the concentration used, still contained a sufficient amount of protein to mask the detection of all required amino acids, or that the organisms could synthesize most of the amino acids at a slow rate. Subsequent experiments with stock 47.8, in which the concentration of NDF was decreased to 1 mg./ml. (w/v) confirmed the former hypothesis. The final population in the control (the complete amino acid medium) decreased thereby from an average of about 10,000 organisms/ml. to about 4000/ml. (Fig. 1). However, the modification allowed for a more complete analysis of the amino acid requirements. Data obtained from these experiments are presented in Table 5. The single omission of each of the seventeen amino acids showed that eleven of them: tryptophan, methionine, threonine, isoleucine, leucine, lysine, histidine, arginine, serine, phenylalanine and tyrosine were absolute growth requirements for stock 47.8, for in each case no growth was obtained in the third serial subculture.

The need for an exogenous source of valine, proline and glycine for optimum growth is also clear (Table 5). In the first transfer of *Paramecium aurelia* from a stock culture to a valine-less medium, growth was limited to 49% of the control followed by the stabilization of growth, through seven transfers at a level of 31% of the control. A more gradual growth decline was apparent in a medium lacking proline or glycine, and the number of organisms became stabilized at 50–40% of the control through seven subcultures. The omission of alanine, glutamic acid, or aspartic acid, singly, resulted in some growth decline in the initial transfers, but then all such cultures stabilized at numbers equal to or greater than the control. When proline, glycine, alanine, glutamic acid and aspartic acid together were omitted from the medium, growth was limited to about 40% of that obtained in the complete medium.

Table 4. *A comparison of two stocks of Paramecium aurelia, var. 4, in a complete medium and in media lacking in amino acids*

The complete medium was composed of the basal medium given in Table 1 plus the amino acid mixture given in Table 2 minus hydroxyproline and cystine. All media contained 4 mg./ml. of the non-dialysable yeast fraction (NDF).

Medium	Stock 47.8. No. of 5-day serial subcultures				Stock 51.7. No. of 5-day serial subcultures			
	1	2	3	4	1	2	3	4
	Percentage organisms/unit volume of medium as compared with complete medium taken as 100%							
Complete	100	100	100	100	100	100	100	100
Complete								
Minus phenylalanine	50	43	41	78	89	86	45	81
Minus tyrosine	69	24	27	0	81	17	0	—
Minus tryptophan	65	30	45	63	89	62	65	100
Minus methionine	19	0	10*	0	45	0	17*	0
Minus threonine	42	38	32	50	78	69	45	57
Minus isoleucine	31	38	36	50	78	24	37	43
Minus leucine	54	43	52	53	100	45	50	65
Minus lysine	85	73	67	78	74	100	62	89
Minus histidine	58	70	47	0	96	100	89	89
Minus arginine	81	51	54	50	74	96	62	51
Minus valine	62	73	54	75	81	79	70	81
Minus serine	38	0	30*	16	59	0	47*	0
Minus alanine, glycine, proline, aspartic acid and glutamic acid	44	48	49	45	87	79	78	74

\* Reinoculated.

#### *B-vitamin requirements*

The replacement of the complex proteose-peptone component of the earlier medium by amino acids made a re-examination of the B-vitamin requirements for *Paramecium aurelia* possible. Initial experiments with stock 47.8 in the amino acid medium, using high NDF concentrations, clearly showed that this stock required folic acid, riboflavin and thiamine. No indication for the requirement of choline, biotin or vitamin B<sub>12</sub> was apparent in any of the

Table 5. *Amino acid requirements of Paramecium aurelia, var. 4, stock 47.8*

The complete medium was composed of the basal medium given in Table 1 plus the amino acid mixture given in Table 2 minus hydroxyproline and cystine. All media contained 1 mg./ml. of the non-dialysable fraction (NDF).

Medium	No. of 5-day serial subcultures			
	1	2	3	4
	Percentage organisms/unit volume of medium as compared with complete medium taken as 100 %			
Complete	100	100	100	100
Minus tyrosine	35	0	—	—
Minus phenylalanine	54	9	0	—
Minus tyrosine minus phenylalanine	43	3	0	—
Minus tyrosine + 3 × phenylalanine	40	18	0	—
Minus tryptophan	32	9	0	—
Minus methionine	24	0	—	—
Minus threonine	30	6	0	—
Minus isoleucine	30	15	0	—
Minus leucine	35	9	0	—
Minus lysine	59	18	0	—
Minus histidine	32	3	0	—
Minus arginine	70	12	0	—
Minus serine	12	0	—	—
Minus valine	49	33	34	33*
Minus proline	78	64	57	51*
Minus glycine	73	45	31	37*
Minus alanine	92	85	100	100*
Minus aspartic acid	57	70	100	100*
Minus glutamic acid	76	97	100	100*
Minus proline, alanine, glycine, aspartic acid and glutamic acid	70	51	43	43*

\* Carried through three more transfers without change in the final population density.

experiments. That pantothenate, nicotinamide and pyridoxal might be required was, however, suggested by the inconsistent results which at times showed definite suboptimal growth in the absence of any one of these three vitamins. When using a lower NDF concentration (0.75 mg./ml.) a clearer response to the absence of these vitamins was given. Table 6 shows the results obtained upon the omission of each of these factors both singly and in combination. In addition to folic acid, riboflavin and thiamine, pantothenic acid and nicotinamide are essential metabolites for stock 47.8, while a clearer indication for the requirement or perhaps the suboptimal synthesis of pyridoxal is apparent.

#### *Inhibition of growth by 2, 6-diaminopurine*

Hamilton (1953) reported that *Paramecium aurelia*, stock 51.7, grown on cultures of living *Aerobacter aerogenes*, directly utilized adenine, guanine and 2,6-diaminopurine for polynucleotide synthesis. Also a requirement for guanylic acid and cytidylic acid by *Paramecium multimicronucleatum* in axenic culture was reported by Johnson (1952). Young (1955) and Young &



van Wagtendonk (1956) found that *P. aurelia*, stock 51.7 (s), required a uridine diphosphate conjugate for growth in an axenic medium. In the course of testing several nucleic acid derivatives for growth-promoting activity it was observed that 2,6-diaminopurine inhibited the growth of organisms of stocks 51.7 and 47.8 in the range of 1–4  $\mu\text{g./ml.}$  The response of these stocks to increasing concentrations of 2,6-diaminopurine are given in Fig. 2.

Table 6. *B-vitamin requirements of Paramecium aurelia, var. 4, stock 47.8*

The complete medium was composed of the basal medium given in Table 1 plus the amino acid mixture given in Table 2. All media contained 0.75 mg./ml. of the non-dialysable yeast fraction (NDF).

Medium	Number of 5-day serial subcultures			
	1	2	3	4
	Percentage organisms/unit volume of medium as compared with the complete medium taken as 100 %			
Complete	100	100	100	100
Minus folic acid	0	0*	10*	0
Minus riboflavin	34	0	29*	0
Minus thiamine	100	14	0	100*†
Minus pantothenic acid	54	12	0	65*‡
Minus nicotinamide	46	17	0	86*‡
Minus pyridoxal	88	35	49	34§
Minus biotin	79	93	100	100§
Minus choline	94	86	100	100§

\* Reinoculated. † No growth in the next subculture. ‡ No growth in the third subculture. § Carried through three additional subcultures without change in the final population density.

*Growth rate differences between stocks 47.8, 32.7, 51.7, 51.8, and 29.7 in axenic culture*

Differences in growth rate, and thus in final population densities, were immediately obvious among the different stocks when cultivated in an axenic medium. All of the stocks examined have been carried through more than 100 successive transfers and a direct comparison of the growth rates has been made from time to time. The differences in growth rate persist even in the most complex axenic medium employed. The rate of growth in an identical medium always followed the pattern: stock 47 > stock 32 > stock 51 > stock 29. Table 7 shows a direct comparison of these stocks carried simultaneously in the complete amino acid medium containing the optimum concentration of NDF. Both mating types of stock 51 were compared to determine whether a mating type difference might possibly effect a growth rate difference. An interesting comparison can be made between the difference in growth rate of these five stocks and the reported micronuclear chromosome numbers (Dippell, 1954) for these same stocks. The average rate of growth of the stocks parallels roughly the chromosome number. No difference in growth rate was apparent between the two mating types 7 and 8.

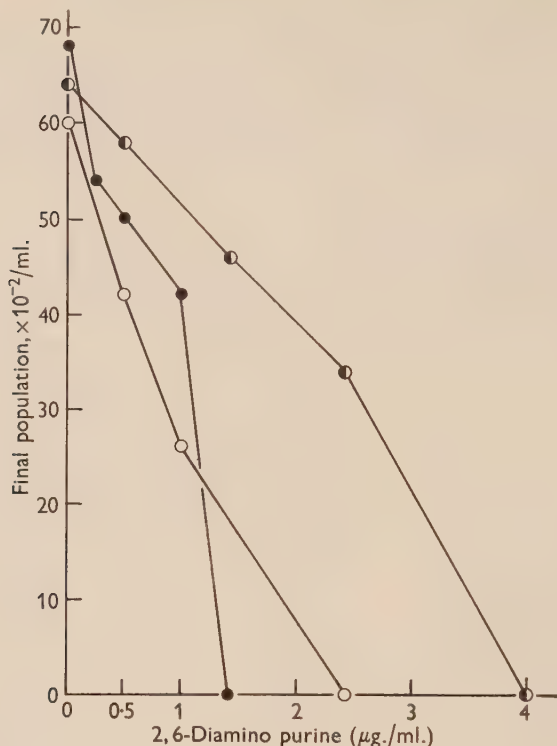


Fig. 2. Inhibition of the growth of *Paramecium aurelia* in various axenic media by 2,6-diaminopurine. ●, Stock 47.8 in a proteose peptone-whole yeast extract (4 mg./ml.) medium; ○, stock 47.8 in a proteose peptone + B-vitamins + NDF (2 mg./ml.) medium; ◐, stock 51.7 in a proteose peptone + B-vitamins + NDF (2 mg./ml.) medium. The final population was determined 5 days after inoculation.

Table 7. Differences in final population densities in successive subcultures and in chromosome numbers of five stocks of *Paramecium aurelia*, var. 4

The corresponding subcultures of the different stocks were grown simultaneously in the basal medium plus the amino acid mixtures shown in Table 2 and with a concentration of 1.5 mg./ml.

Stock	No. of 5-day serial subcultures								Mean	Chromosome no.
	1	2	3	4	5	6	7	8		
	Final population $\times 10^{-2}$ organisms/ml.									
47.8	66	90	100	64	76	69	68	60	75	47-51
32.7	—	—	—	40	31	30	32	32	33	44-46
51.7	19	27	38	26	29	20	28	25	27	41-45
51.8	—	29	32	22	20	27	21	25	25	41-45
29.7	15	16	25	21	16	21	16	18	19	33-37

## DISCUSSION

Specific amino acid requirements have been reported for only two other ciliates, *Tetrahymena pyriformis* (Kidder & Dewey, 1951) and *Glaucoma scintillans* (Fuller, 1948). Although the basal requirements of *Paramecium*

*multimicronucleatum* and *P. aurelia* appear to be the same in crude medium, Johnson (1952) was unable to substitute free amino acids for Proteose-peptone in his sterile medium for *P. multimicronucleatum*. The reason for this might be due to the use of an inadequate mixture of amino acids or to the low concentration of B-vitamins in the medium. Another factor which has contributed to the successful establishment of *P. aurelia* in an amino acid medium is the supplement of the essential sterol in a purified uniformly available form.

The replacement of Proteose-peptone with amino acids in the axenic medium for *Paramecium aurelia* constitutes a major step toward the goal of obtaining this organism in a complete chemically defined medium. The fact that growth falls to zero within the first few subcultures when any one of the reported eleven essential amino acids is absent from the medium indicates the lack of ability to synthesize these acids. The more than 75 % diminution of growth in the absence of valine suggests that this amino acid can be synthesized only at a limiting rate by *P. aurelia*; this may also be the case for proline and glycine. A comparative nutritional study of several vertebrates has shown that valine is required by nearly all. Proline has been reported to be required by *Trichomonas foetus* (Weiss & Ball, 1947), *Glaucoma scintillans* (Fuller, 1948), glycine by *Trichomonas foetus*, and by *Aedes aegypti* (Goldberg & de Meillon, 1948). The absolute requirement for serine by *P. aurelia* is paralleled in *Tetrahymena pyriformis*, strain E, as reported by Elliott (1949). A closer examination of the proper concentrations and metabolic interrelationships of the amino acid requirements of *Paramecium aurelia* must await the fuller understanding of other metabolites such as the carbon sources, salts, and other growth factors.

The inability of *Paramecium aurelia* to oxidize phenylalanine to tyrosine is concluded from the fact that an exogenous source of both amino acids is essential for continued growth. Most animals can meet their tyrosine needs when supplied with a source of phenylalanine, although apparently the reverse reaction does not occur; only tyrosine will spare phenylalanine in such cases. Until recently a one-step oxidation was postulated for the conversion of phenylalanine to tyrosine. However, studies of auxotrophic mutants of *Escherichia coli*, K12, strain W (Simmonds, Dawling & Stone, 1954) strongly support the view that the conversion of phenylalanine to tyrosine is indirect, the two amino acids being synthesized from a common precursor by reversible reactions.

The essential NDF component of the present medium remains complex. It contains polysaccharides, some protein, and apparently some non-dialysable vitamin conjugates. The yeast fraction contributes essential nucleic acid derivatives (Young, 1955; Young & van Wagtendonk, 1956). This is supported by Johnson's (1952) demonstration of the need for guanylic and cytidylic acid in *Paramecium multimicronucleatum*.

Little is known of the requirements for recognized vitamins in ciliates other than *Tetrahymena pyriformis*. Kidder & Dewey (1951) recorded that thiamine, riboflavin, the vitamin B<sub>6</sub> group, pantothenic acid, folic acid and nicotinamide are required for *T. pyriformis*. *Colpoda duodenaria* (Tatum, Garnjobst & Taylor, 1942; Garnjobst, Tatum & Taylor, 1943) requires relatively high

concentrations of thiamine, riboflavin, pantothenic acid, nicotinamide and pyridoxine. *Paramecium aurelia* requires thiamine, riboflavin, pantothenic acid, nicotinamide and folic acid. The organism apparently can synthesize pyridoxal (and the other members of the B<sub>6</sub> group) at a suboptimal rate.

The observations on the growth inhibition of two stocks of sterile *Paramecium aurelia* by 2,6-diaminopurine in a very low concentration are in direct contradiction with those reported by Hamilton (1953) for *P. aurelia* grown in living *Aerobacter aerogenes* cultures. Although further work must be done to clarify the phenomenon, this single observation emphasizes the need for employing axenic cultures for many of the studies designed to analyse protozoan metabolism.

The differences in growth rate of stocks 47.8, 32.7, 51.7, 51.8 and 29.7 cannot be satisfactorily explained. The difference is evident in each medium investigated and can therefore not be due to an unknown essential metabolite for the slower growing stocks. If the chromosome differences are indeed due to aneuploidy, as suggested by Dippell (1954), and if the apparent correlation between the chromosome number of the stocks and their rate of growth is valid, the following explanation might be offered. The stocks would differ by having different numbers of whole gene sets through the duplication of whole chromosomes or of parts thereof, rather than differing at the same single locus, or at a few loci through mutations. The differences in growth rates might then be explained by a greater or lesser synthetic capacity according to the number of like sets of genes present through ploidy in part of the genome.

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## Quantitative Aspects of the Enhancing Action of Eperythrozoon on the Pathogenicity of Mouse Hepatitis Virus

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**SUMMARY:** The enhanced pathogenicity of mouse hepatitis virus (MHV 1) in mice infected with *Eperythrozoon coccoides* is shown to result from the increased probability of virus particles actively infecting cells in the presence of the blood parasite. The ratio ( $\alpha$ ) of the probability that a virus particle will infect a cell in the absence of *E. coccoides* to the probability of its doing so in the presence of *E. coccoides* has been estimated by two independent methods. One method depends upon the increased infectivity of dilute suspensions of MHV 1 for mice pretreated with *E. coccoides* and gives values for  $\alpha$  varying between 0.34 and 0.77, with a mean of 0.5. The other method, in which relatively large doses of MHV 1 are used, depends upon estimating the slope of the regression line of survival time on time of infection with *E. coccoides*, and yields a value for  $\alpha$  of 0.49, with 0.95 probability limits of 0.37 and 0.61. The enhancing effect of *E. coccoides* on the pathogenicity of MHV 1 is attributed to the parasite increasing the ratio of active to latent infections of cells by the virus.

Mouse hepatitis virus (MHV 1) produces a mild, rarely fatal, hepatitis in weanling VS mice but when such mice are simultaneously infected with *Eperythrozoon coccoides* (a normally harmless blood parasite of mice) fatal hepatitis is invariably produced (Gledhill, Dick & Andrewes, 1952; Niven, Dick, Gledhill & Andrewes, 1952; Gledhill, Dick & Niven, 1955). When mice are first infected with virus and then inoculated a day or so later with a mixture of virus and *E. coccoides*, the majority survive. This decreased mortality which is produced by a prior inoculation of MHV 1 cannot be ascribed to humoral antibody (Gledhill & Dick, 1955; Dick, Gledhill & Niven, to be published). To explain this observation and observations relating to mice which are virus carriers, Gledhill & Dick (1955) advanced the hypothesis that when MHV 1 suspension is introduced intraperitoneally into weanling mice, the virus produces active infections of some liver cells and latent infections of other liver cells. By active infections of cells are meant infections in which cells liberate virus and are probably destroyed; and by latent infections are meant infections in which the cells do not forthwith liberate virus but become resistant to subsequent infection. It was suggested that prior or concomitant infection with *E. coccoides* enables active rather than latent infections of cells to be produced and thereby gives rise to fatal hepatitis. The mild regressive disease produced by MHV 1 in the absence of *E. coccoides* is due to latent infections of many cells.

In this paper it is shown that when mice are inoculated with small doses of *Eperythrozoon coccoides* more mice develop hepatitis when they have been preinoculated with *E. coccoides* than when they are simultaneously infected with virus and blood parasite, and that the survival time of mice is greatly

decreased by previous inoculation with *E. coccoides*. The relationship between survival time and time of inoculation with *E. coccoides* was found to be linear for constant doses of virus and blood parasite. All the results may be explained by the hypothesis that in the absence of *E. coccoides*, MHV 1 produces active and latent infections in about equal proportions of liver cells, while in the presence of *E. coccoides* the virus produces only active infections of liver cells.

In order to make the purpose and methods of this paper more readily understood it is useful to recall that when a high dilution of *Eperythrozoon coccoides* is inoculated into mice, the parasites become abundant in the blood on about the 4th day and, rising to a maximum on about the 6th day, they decline more

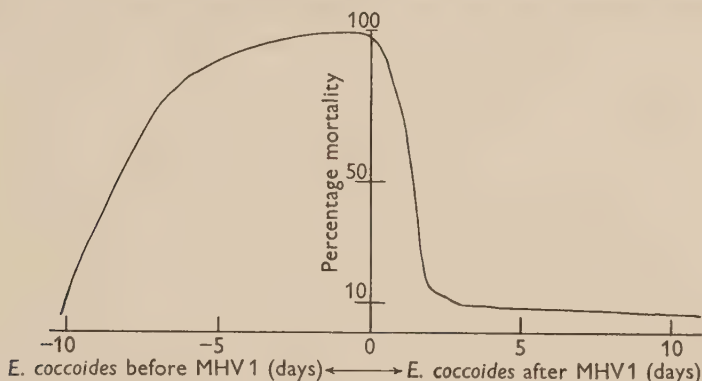


Fig. 1. Mortality for different relative times of inoculating *Eperythrozoon coccoides* and MHV 1.

slowly, becoming very scarce in blood smears after about the 10th day. When *E. coccoides* is inoculated into mice on the same day as virus, fatal hepatitis results. When mice are inoculated with *E. coccoides* more than one day after inoculation with virus, many survive as a result of the development of cellular resistance to the virus. When the inoculation of *E. coccoides* precedes that of virus by 1–5 days the mortality remains at almost 100 % and declines to about 75 % and 10 % when the blood parasite precedes the virus by 7 and 10 days, respectively. This sharp decline in mortality when the inoculation of *E. coccoides* precedes that of virus by more than 7 days is believed to be due to the low concentration of the blood parasite in the mouse tissues during the later phase of virus growth. The mortality curve for different times of inoculation with *E. coccoides*, based on published and unpublished records, is shown in Fig. 1.

#### METHODS

The general methods have been described elsewhere (Gledhill & Andrewes, 1951; Gledhill *et al.* 1952, 1955). Weanling VS mice aged 20–22 days were employed in all experiments. Mice of this strain originated from the virus-susceptible, bacteria-susceptible variant of the Rockefeller strain of albino mice (Webster, 1937) and have been bred since 1949 at the National Institute for Medical Research, Mill Hill. Samples of two pools (pools 1 and 2) of 10 % MHV 1 infected mouse liver spleen suspension which were stored at  $-70^{\circ}$

were used. These pools were titrated intraperitoneally in mice which had been infected 2 days previously with *Eperythrozoon coccoides*. The ID 50 was  $0.2 \times 10^{-4.5}$  ml. for pool 1 virus and  $0.2 \times 10^{-3.9}$  ml. for pool 2 virus as calculated by the method of Reed & Muench (1938) from the proportion of mice with fatal or severe hepatitis at autopsy 7 days after injection of replicate four-fold or less dilutions of virus into groups of 6–10 mice. Experiments to be described yield regression lines (see Fig. 2) from which the ID 50's can be estimated as  $0.2 \times 10^{-4.8}$  ml. for pool 1 and  $0.2 \times 10^{-3.9}$  for pool 2 (see foot of Table 2). Suspensions of *E. coccoides* at dilutions of  $10^{-7}$  were used to infect mice. This dilution was used to exclude the possibility that MHV 1 might occasionally be present in the inoculum as a result of its liberation by *E. coccoides* in sporadic carriers amongst the mice which provided the suspension of this parasite (Gledhill & Dick, 1955).

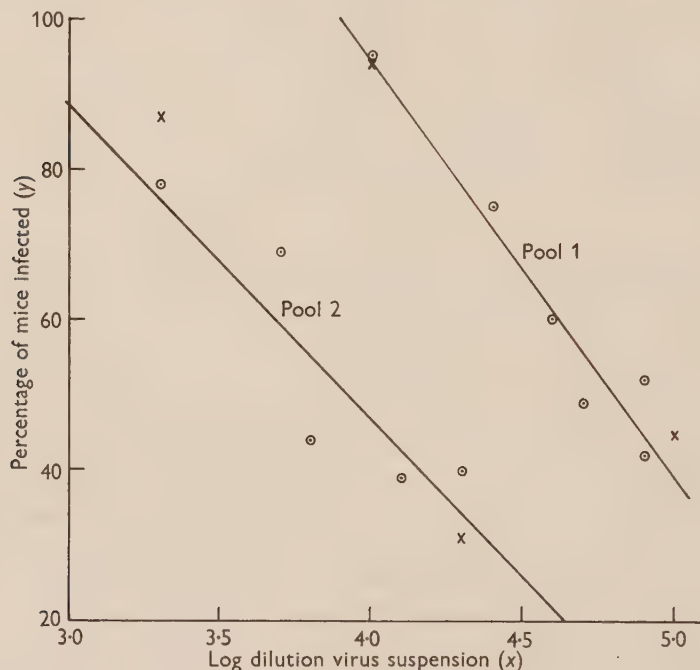


Fig. 2. Regression of percentage mice infected ( $y$ ) on log dilution ( $x$ ). ○, points used for determining regression lines (see Table 2); ×, independent points from confirmatory experiments. Regression lines calculated from ○ points: Pool 1 virus— $Y = 317 - 55.6x$ ; Pool 2 virus— $Y = 216 - 42.2x$ .

## RESULTS

### *Increased infectivity of dilute MHV suspensions in the effective presence of Eperythrozoon coccoides*

The hypothesis that infection with *Eperythrozoon coccoides* enables MHV 1 virus to produce active rather than latent infections of cells suggested that if a minimal dose of virus were inoculated the proportion of mice infected would be



increased by the presence of *E. coccoides* in effective concentration at the time of inoculation of virus. For, under these conditions, cells are actively infected, whereas in the absence of *E. coccoides* some infections of cells are latent and these would not initiate progressive hepatitis. The increased infectivity due to *E. coccoides* results only from its effective presence at the time of infection with MHV 1 and during the first cycle of virus growth. In contrast, the increased severity of MHV 1 hepatitis in mice infected with *E. coccoides* results mainly from the effective presence of the parasite during the middle and late cycles of virus growth, when infection is spreading to the majority of liver cells. In mice inoculated with *E. coccoides* suspension diluted to  $10^{-4}$ , the parasite is not abundant in the blood for 4 or 5 days. Moreover, the experiment detailed later (pp. 299–302) and other experiments show that *E. coccoides* does not enhance the growth of MHV 1 virus until the parasite has become abundant in the blood. Thus we may use the expression ‘the effective presence of *E. coccoides*’ to mean its presence in sufficient concentration to enhance the growth of virus. It was considered that if a high dilution of *E. coccoides* were inoculated simultaneously with virus or preceded the virus by 2 days, *E. coccoides* would not be effectively present during the first cycle of virus multiplication and so would not increase the infectivity of the virus suspension. Experiments were devised to compare the infectivity of a suitable dose of MHV 1 virus in mice infected with *E. coccoides* 5–7 days before inoculating them with virus (termed ‘mice preinfected with *E. coccoides*’) and in mice infected simultaneously or not more than 2 days previously with *E. coccoides* (termed ‘mice simultaneously infected with *E. coccoides*’). Doses of virus were chosen which would infect only a proportion of the inoculated mice, and the results were determined by the specific deaths and by the lesions shown by the remainder at autopsy 7 days after infection with virus. Since all mice had been inoculated with *E. coccoides*, infected mice of both groups developed severe hepatitis with gross lesions and were readily distinguishable from mice which failed to develop active infection with virus. Experiments were concluded by sacrificing survivors on the 7th day after virus inoculation rather than by basing results on the specific mortalities within a somewhat longer time because in mice treated with *E. coccoides* 7 or even 5 days before virus, severe hepatitis does not invariably end fatally, as already noted (see Fig. 1). Such experiments yielded estimates of the increased infectivity of virus in mice pretreated with *E. coccoides*.

Two independent experiments were carried out with virus of each pool and the results are shown in Table 1. In each experiment the infectivity of virus suspension was higher for mice pretreated with *Eperythrozoon coccoides* by amounts varying from 6 to 26 %. Two of the differences are statistically significant and the difference of the sum of the experiments is highly significant. Before concluding that virus suspension has increased infectivity for mice pre-treated with *E. coccoides*, another possible explanation of the observed differences must be considered. It should be recalled that infection with *E. coccoides* liberates MHV 1 in about 10 % of VS mice and that the liberated virus produces hepatitis in them (Gledhill & Dick, 1955). It is therefore possible that the observed differences might be due to spontaneous MHV 1

hepatitis having become observable in a higher proportion of mice which had been infected with *E. coccoides* 5–7 days before giving the virus, as compared with mice simultaneously infected with *E. coccoides* + virus. This was investigated in the following experiment in which the proportions of mice with hepatitis at autopsy 7 and 12 days after infection with *E. coccoides* were compared. Fifty of 100 mice were inoculated with *E. coccoides*, and 5 days later the other 50 were similarly inoculated. All the mice were autopsied after a further 7 days and in each group of 50 there were 4 with hepatitis such that they would have

Table 1. *Higher infectivity of dilute virus MHV 1 suspension for mice pre-treated with Eperythrozoon coccoides*

Expt. no.	Virus pool	-log dose	Infectivity ratio, mice infected/total mice				
			Pre-treated with <i>E. coccoides</i>	Simultaneously- treated with <i>E. coccoides</i>	Probability of difference occurring by chance		
					$\chi^2$	$n=1$	Percentage difference (%)
1	P1 (log ID 50 4.8)	4.7	46/68 (68 %)	33/68 (49 %)	4.35	<0.05	19
2	P1 (log ID 50 4.8)	5.0	31/61 (51 %)	33/73 (45 %)	0.22	>0.5	6
3	P2 (log ID 50 3.9)	4.1	44/81 (54 %)	35/89 (39 %)	3.25	>0.05	15
4	P2 (log ID 50 3.9)	4.3	26/46 (57 %)	18/58 (31 %)	5.82	<0.02	26
Total			147/256 (57.4 %)	119/288 (41.3 %)	13.43	<0.001	16

been falsely accepted as infected in titrations of virus. This single experiment would suggest that carriers of MHV 1 virus influence the two methods of titration to an equal extent. Moreover, even supposing that 10 % of mice developed hepatitis 12 days after infection with *E. coccoides* and no mice developed it within 7 days, an allowance for this hypothetical situation can be made in figures presented in Table 1. When this is done the infectivity ratio in mice pre-treated with *E. coccoides* becomes 135/256 instead of 147/256, and, the ratio for mice not pretreated with *E. coccoides* remaining 119/288, the difference becomes 11 % instead of 16 %. The probability of this difference occurring by chance is 0.01 ( $\chi^2=6.64$ ). It may therefore be concluded that the difference between the two methods of titration is a real one and is attributable to the increased infectivity of virus suspension for mice pre-treated with *E. coccoides*.

The difference between the proportion of mice demonstrably infected following inoculation of the same number of virus particles into simultaneously-treated and pretreated mice was determined. This enabled an estimate to be made of the greater number of virus particles needed to produce the same increase in the proportion of mice infected. This was done by infecting with dilutions of virus mice which had been inoculated with *Eperythrozoon coccoides* within 2 days and determining the proportion which developed hepatitis, as in previous experiments. These determinations (Table 2) represent the decreasing percentage of infected mice with increase of log dilution of virus suspension and are shown graphically in Fig. 2, together with the regression lines of percentage of mice infected on log dilution of virus calculated from them. It will be seen that the observations tend to be distributed linearly and that the

regression lines for the two pools are nearly parallel. The slopes of the lines, viz. 55.6 and 42.2 respectively, represents the increase in percentage infected per unit log dilution. The percentage higher infectivities of pool 1 and pool 2 virus for pre-treated mice were 19 and 15 % respectively (Table 1, Expts. 1 and 3). To get 19 % increased infectivity with pool 1 virus would require a decrease of log dilution of 19/55.6 or 0.34, and to get 15 % increased infectivity with pool 2 virus would require a decrease of log dilution of 15/42.2 or 0.36.

Table 2. *Regression of infectivity for mice simultaneously treated with Eperythrozoon coccoides on log dilution of virus*

Pool 1 virus			Pool 2 virus		
Log dilution of virus ( <i>x</i> )	Infectivity ratio		Log dilution of virus ( <i>x</i> )	Infectivity ratio	
	Mice infected	Percentage ( <i>y</i> )		Mice infected	Percentage ( <i>y</i> )
	Mice in group			Mice in group	
4.9	25/60	42	4.3	25/62	40
4.9	40/77	52	4.1	35/89	39
4.7	33/68	49	3.8	35/80	44
4.6	59/98	60	3.7	42/61	69
4.4	45/60	75	3.3	47/60	78
4.0	57/60	95			

From the above data the regression lines have been calculated for each virus pool and are as hereunder: Regression of percentage of mice infected ( $y$ ) on log dilution ( $x$ ) for pool 1 virus  $Y = 317 - 55.6x$ ; Regression of percentage of mice infected ( $y$ ) on log dilution ( $x$ ) for pool 2 virus  $Y = 216 - 42.2x$ . Substituting 50 % for  $y$  in these equations gives ID 50's 0.2 ml.  $\times 10^{-4.8}$  for pool 1 virus and 0.2 ml.  $\times 10^{-3.93}$  for pool 2 virus.

In a repeat experiment for each virus pool, the same assumptions and procedures were followed but the regression lines were drawn through two points based on entirely new data. About 150–200 mice were divided at random into three approximately equal groups. One group (pretreated group) was inoculated with *Eperythrozoon coccoides* 6 days before virus inoculation, and the other two groups (simultaneous groups) were treated with the parasite one day before they were inoculated with virus. A dose of virus, estimated as sufficient to infect about 30–50 % of mice, was inoculated into the group of pre-treated mice and into one group of simultaneously-treated mice. Ten times this dose of virus was inoculated into the other group of simultaneously-treated mice. The infectivities for the pretreated and simultaneously-treated mice in the experiment with pool 1 and pool 2 virus have already been presented in Table 1 (Expts. 2 and 4). The infectivity rate for the simultaneously-treated mice inoculated with ten times the dose of virus was 65/69 (94 %) for pool 1 virus and 46/53 (87 %) for pool 2 virus. These results, expressed as percentages, are summarized below:

	Pool 1 virus		Pool 2 virus	
	Log virus dilution	% mice infected	Log virus dilution	% mice infected
Pretreated mice	5.0	51	4.3	57
Simultaneously-treated mice	5.0	45	4.3	31
Simultaneously-treated mice	4.0	94	3.3	87



It will be seen that for pool 1 virus, an increase of log dilution by 1 log unit lowered the infectivity from 94 to 45 %, that is, by 49 %. Pretreatment of mice with *E. coccoides* raised the infectivity from 45 to 51 %, i.e. 6 % and this percentage increase is therefore equivalent to decreasing the log dilution by 6/49 or 0.12 in simultaneously-treated mice. Similarly, for the pool 2 virus one log unit dilution lowered the infectivity from 87 to 31 %, i.e. 56 %, and pre-treatment with *E. coccoides* raised the infectivity from 31 to 57 %, i.e. 26 %. This percentage increased infectivity in pretreated mice is equivalent to decreasing the log dilution 25/56 or 0.46 in simultaneously-treated mice. Thus the four independent results for the decrease in log dilution in simultaneously-treated mice to give the same infectivity as that for pretreated mice are:

Pool 1 original expt.	0.34 (= log 2.2)
Pool 1 second expt.	0.12 (= log 1.3)
Pool 2 original expt.	0.36 (= log 2.3)
Pool 2 second expt.	0.46 (= log 2.9)
Mean	0.32 (= log 2.1)

Although it might appear that the closeness to the mean of the results in the original experiments with virus of both pools resulted from the greater accuracy of the regression of infectivity on log dose in these experiments, it will be seen in fact that the wide deviations from the mean in the second experiments resulted from wide differences of infectivity between pretreated and simultaneously-treated mice in these experiments (6 and 26 %). Since the anti-log of 0.32 is 2.1, it is concluded that about twice as many virus particles must be inoculated into simultaneously-treated mice as into pre-treated mice to infect an equal proportion of them and that the estimate of 'twice as many virus particles' varied in the four experiments from 1.3 to 2.9 times as many virus particles. Equivalently, we may say that if the probability of a virus particle actively infecting a liver cell of a mouse in which *E. coccoides* is not effectively present is  $p_1$  and its probability of doing so in a mouse in which the parasite is effectively present is  $p_2$ , the ratio  $p_1/p_2$ , termed  $\alpha$ , has an estimated value of 1/2.1, i.e. about 0.5 and varied in the experiments from 1/2.9 to 1/1.3 which is from 0.34 to 0.77. Statement of the result in terms of probability relates to the probability of a virus particle producing a cell-virus union which is active and does not imply that *E. coccoides* increases the infectivity of virus by altering the virus itself. Indeed, it is more likely that the host cells are altered by infection with the parasite.

*Regression of survival time of virus-infected mice on the time of infecting them with Eperythrozoon coccoides*

It would be expected that the greater probability of virus particles infecting cells in the presence of *Eperythrozoon coccoides* would also manifest itself by the swifter spread of virus throughout the liver cells of mice in which *E. coccoides* was effectively present. Qualitatively it has been repeatedly observed that mice pretreated with *E. coccoides* die of hepatitis following an injection of virus sooner than mice which receive the blood parasite at the same time as



the virus. This observation can be put on a quantitative basis by making the assumptions that, following inoculation of virus, the number of virus infected cells increases logarithmically with time until such a number of cells have been infected that the mouse inevitably dies of hepatitis. With these assumptions it can be shown that the average survival time ( $y_1, y_2, y_3$ , etc.) of groups of mice inoculated with the same dose of *E. coccoides* at times ( $x_1, x_2, x_3$ , etc.) measured from the time of inoculation of a constant dose of virus yield points which fall on a line and that the slope of this line is  $(1 - \alpha)$ , where  $\alpha$  is, as before, the ratio of the probability ( $p_1$ ) of a virus particle infecting a liver cell in the absence of *E. coccoides* to its probability ( $p_2$ ) of doing so in the presence of *E. coccoides*. The derivation of the equation of this line is as follows.

Suppose that an inoculated dose of virus infects  $N_0$  mouse liver cells and that after time  $t$  (measured from the time of infection with virus), the number of infected liver cells is  $N$ . In the case of a mouse in which *E. coccoides* is effectively present at the time of infection with virus ( $t=0$ ), optimal virus growth will occur from the beginning and the number of infected cells  $N$  at time  $t$  will be given by  $N = N_0 e^{kt}$ , where  $k$  is a constant and  $e$  is the base of natural logarithms. If *E. coccoides* is not effectively present at the commencement of virus growth and if the probability of a virus particle infecting a cell in the absence of *E. coccoides* is  $\alpha$  times the probability of its infecting a cell in the presence of this parasite, the number of cells infected at time  $t$  in the absence of the parasite will be given by  $\alpha N_0 e^{\alpha kt}$ . Consider a group of mice inoculated with a dose of virus at time zero and with a dose of *E. coccoides* at time  $x$  and suppose that from time zero to time  $t'$  there are insufficient blood parasites present to influence virus growth and that after time  $t'$  the number of blood parasites is sufficient to give optimum virus growth. Then, the number of infected cells  $N_1$  at time  $t_1$  is  $\alpha N_0 e^{\alpha kt'}$  and the number  $N$  infected at time  $t$  ( $t > t'$ ) is  $N_1 e^{k(t-t')}$ , that is  $\alpha N_0 e^{\alpha kt' + k(t-t')}$ . Suppose that when a certain critical number of cells ( $N_2$ ) have been infected (at time  $t''$ ) the mouse is predetermined to die and that death occurs, on the average, after a further time  $A$ . Then,  $N_2 = \alpha N_0 e^{\alpha kt' + k(t''-t')}$ . If the observed average survival time for the group of mice is  $y$ , then  $t'' = y - A$ . If  $B$  is the average time from the inoculation of *E. coccoides* until it enhances virus growth, then  $t' = x + B$  and thus  $N_2 = \alpha N_0 e^{\alpha k(x+B) + k(y-A-x-B)}$ . Taking logarithms, rearrangement of terms gives the relationship  $y = \left[ \frac{1}{k} \log \frac{N_2}{\alpha N_0} + A + B(1 - \alpha) \right] + (1 - \alpha)x$ . The first three terms of the right side (enclosed in square brackets) are constant for constant doses of virus and *E. coccoides* and may be replaced by the symbol  $a$  to give the equation  $y = a + (1 - \alpha)x$ . There are obvious limitations to the values of  $x$  for which this equation may be expected to be valid. If the inoculation of *E. coccoides* follows the inoculation of virus by more than 1 day (i.e. if  $x > 1$ ) the virus would grow sufficiently to make many mice resistant before the parasite became effective and the equation could not be applicable (see Fig. 1). In the other direction, if *E. coccoides* were inoculated into groups of mice more than 4 days before the virus (i.e. groups in which  $x < -4$ ), the parasite would exert its full effect in all the groups from the time the virus was first inoculated: for example, three groups of mice inoculated with the parasite, 5, 6 and 7 days before virus inoculation, all would have the same minimal survival time and the equation connecting their average survival times with times of inoculation of *E. coccoides* would be a line parallel with the  $x$ -axis in place of the line of slope  $(1 - \alpha)$ . The range from  $x = -3$  to  $x = 0$  would be expected to lie well within the zone of validity of the equation  $y = a + (1 - \alpha)x$ .

The following experiment was carried out in order to test whether the regression of survival time on time of inoculation of *Eperythrozoon coccoides*

is linear, and to evaluate  $\alpha$ . Pool 2 virus suspension was diluted to give 80 ID 50/mouse dose (0.2 ml.), distributed in four bottles each containing 12 ml. and stored at  $-70^{\circ}$ . Two hundred and ten mice were inoculated with  $10^{-3}$  dilution of spleen mince from an *E. coccoides*-infected mouse which showed no macroscopic evidence of hepatitis; the inoculated mice were then randomly divided into four groups each of 50 mice, leaving 10 mice as controls. The first group of 50 *E. coccoides*-treated mice were forthwith inoculated from a bottle

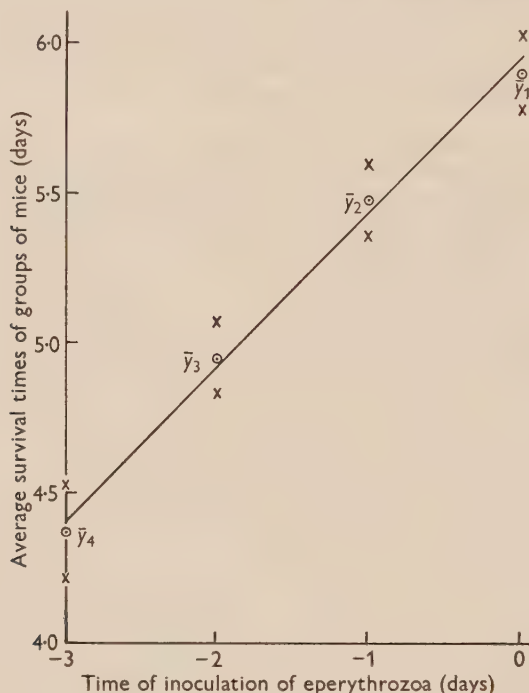


Fig. 3. Regression of mouse survival time ( $y$ ) on time of infection with eperythrozoa ( $x$ ).  $\bar{y}_1$ , mean survival time of mice inoculated with eperythrozoa at time  $x=0$ ;  $\bar{y}_2$ , mean survival time of mice inoculated with eperythrozoa at time  $x=-1$ ;  $\bar{y}_3$ , mean survival time of mice inoculated with eperythrozoa at time  $x=-2$ ;  $\bar{y}_4$ , mean survival time of mice inoculated with eperythrozoa at time  $x=-3$ . All indicated by  $\odot$ . Standard errors of each mean ( $\bar{y}_1$ ,  $\bar{y}_2$ , etc.) indicated by  $\times$ . Regression line  $Y=5.94+0.51x$  calculated from survival times of individual mice.

of virus suspension. For this group of mice (group 1)  $x$ , the time of inoculation of *E. coccoides*, was zero. Exactly 24 hr. afterwards the second group of treated mice was similarly inoculated with virus suspension and for this group  $x=-1$ . The third and fourth groups of mice were similarly inoculated with virus suspensions 48 and 72 hr. after treatment with *E. coccoides* suspension; for these  $x$  was equal to  $-2$  and  $-3$  respectively. The number of mice found dead of hepatitis at each 12 hr. interval after receiving virus was noted; the results are shown in Table 3. Using the time of death of each mouse ( $y$ ) and the time when it was inoculated with *E. coccoides* ( $x$ ) (both measured from the time of virus inoculation) for determining the regression of  $y$  on  $x$ , the following line was

Table 3. *Regression of survival time on time of infection with Eperythrozoon coccoides*

Both times measured from time of infection with virus.

Time of inoculation with <i>E. coccoides</i> ( <i>x</i> in days from virus inoculation)	No. of mice dead of hepatitis at times ( <i>y</i> ) in days from time of virus inoculation												No. of effective mice in group	No. of non-specific deaths	No. of mice which survived	Mean survival time calculated from observations ( <i>y</i> in days)	Standard error ( $\sigma$ ) of mean survival time in days	Mean survival time calculated from regression line ( <i>Y</i> )*
	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5							
1	0	0	0	0	1	13	12	9	5	4	6	50	0	0	5.90	$\pm 0.12$	5.94	
2	-1	0	0	0	2	11	9	6	9	10	2	49	1	0	5.48	$\pm 0.12$	5.43	
3	-2	0	1	1	5	13	8	10	4	1	0	44	2	4	4.95	$\pm 0.12$	4.92	
4	-3	1	4	8	13	5	8	1	0	3	1	45	3	2	4.37	$\pm 0.16$	4.41	
Totals		1	5	9	20	30	38	29	22	19	7	8	188	6	6		Dose of MHV 1: 80 ID 50	
																	Dose of <i>E. coccoides</i> : infected spleen since 10 <sup>-3</sup>	
																	* Regression line calculated from data is $Y = 5.94 + 0.51x$ .	

Dose of MHV 1: 80 ID 50  
Dose of *E. coccoides*: infected spleen mice 10<sup>-3</sup>  
\* Regression line calculated from data is  $Y = 5.94 + 0.51x$ .

defined:  $Y = 5.95 + 0.51x$ . This line is illustrated in Fig. 3, together with the four points representing the mean survival times ( $\bar{y}$ ) for each group of mice (shown in Table 3). It will be seen that the points fall close to the line and, in fact, no point is further from the line than half the standard error of the determination of  $\bar{y}$  for the point itself. Thus, the results are consistent with the hypothesis that the relationship between average survival time and the time of inoculation with *E. coccoides* is linear within the limits of  $x$  varying from  $-3$  to  $0$ . The slope of the regression line ( $0.51$ ) can be equated with that of the theoretical line  $(1 - \alpha)$  and the equation so obtained yields a value of  $0.49$  for  $\alpha$  which, it will be recalled, is the ratio of the probability of a virus particle infecting a cell in the absence of *E. coccoides* to its probability of doing so in the presence of the parasite. Moreover, from the data presented in Table 3 the standard error of  $\alpha$  (which is the standard error of the slope of the regression line) is calculated to be  $0.06$  giving  $0.37-0.61$  as the range for  $0.95$  probability of including the true value of  $\alpha$ . It will be recalled that by the first method the mean value of  $\alpha$  was estimated to be  $0.5$  and individual results varied within the limits of  $0.34-0.77$ .

Since the dilution of *Eperythrozoon coccoides* suspension was  $10^{-3}$  instead of  $10^{-7}$  (as more usual) it might have contained MHV 1 virus if the donor mouse had happened to be a virus carrier (Gledhill & Dick, 1955). However, the control mice (uninoculated with virus) sacrificed 7 days after inoculation with *E. coccoides* showed no macroscopic or microscopic evidence of hepatitis. Blood smears were taken daily from these control mice. *E. coccoides* were absent from all smears 1 day after inoculation; it appeared in small numbers in two of the mice 2 days after inoculation, was present in all smears in moderate numbers 3 days after inoculation and reached maximum numbers 4 days after inoculation. As preliminary experiments had shown that *E. coccoides* begins to enhance MHV 1 growth after the parasites are first demonstrable in blood smears but before they reach a maximum, virus growth in the fourth group of mice ( $x = -3$ ) was probably enhanced from the time of virus inoculation.

#### DISCUSSION

The first method of determining  $\alpha$ , the ratio of the probability of a virus particle actively infecting a liver cell in the absence of *Eperythrozoon coccoides* to the probability of its doing so in its presence, depended upon determination of the increased infectivity of MHV 1 virus suspension in mice pre-treated with *E. coccoides*. It might be contended that this increased infectivity was really due to a greater severity of hepatitis in pre-treated mice as a result of which some instances of hepatitis detected in them would have escaped detection if they had been simultaneously treated with *E. coccoides*. Experiments showed that the proportion of simultaneously treated mice which died of hepatitis within 14 days of infection with about one ID<sub>50</sub> dose of virus equals the proportion of mice which showed lesions within 7 days of infection with the same dose of virus. Moreover, mice autopsied on the 7th day practically all showed either severe progressive hepatitis or no evidence of hepatitis. The facts



strongly support the view that mice which develop hepatitis at all develop fatal hepatitis when *E. coccoides* is effectively present in the middle and late stages of virus growth. This excludes the view that pretreatment with *E. coccoides* raises the proportion of mice with hepatitis by making observable some instances of hepatitis which would escape notice in simultaneously-treated mice. The second method of estimating  $\alpha$  clearly yielded results of greater accuracy than the first method. Thus, in the second method (using 200 mice) the range within which lies the 0.95 probability limits for the true value of  $\alpha$  is from 0.37 to 0.61 as compared with a range from 0.34 to 0.77 for the four results of the first method (using about 1200 mice). Nevertheless, the first method is such that there does not appear to be any other way of interpreting the results than by supposing that *E. coccoides* increases the probability of virus particles infecting cells; whereas the result obtained by the second method can equally be interpreted by the supposition that *E. coccoides* causes every infected cell to produce about twice as many virus particles as it would have produced in the absence of the parasite. Within the framework of the hypothesis which suggested the experiments described in this paper, the increased probability that active infection forthwith results from the introduction of the virus particles in the presence of *E. coccoides* is considered to result from the parasite's causing an increase in the proportion of active infections of cells from about 50 % to about 100 %, with a corresponding reduction of latent infections of cells from about 50 % to practically none.

Natural carriers are believed to be mice in which some liver cells are infected with latent virus. Whether their latent cell infections are due to virus resembling the ordinary virus which has been used for the experiments or to a variant virus which produces a higher proportion of latent infections was not determined (Gledhill & Dick, 1955). Since it now appears that the ordinary virus produces only 50 % latent infection of cells, it is unlikely that carriers are mice infected with small doses of such virus but are probably infected with a variant of MHV 1 which produces a proportion of latent infections of cells so much higher than 50 % that infection does not spread throughout the liver in the absence of *E. coccoides*. Preliminary evidence for the existence of less virulent variants of MHV 1 virus has been obtained and infection of unweaned mice with them appears to produce weanling carriers which simulate natural carriers. In the MHV group of viruses it appears as if the virulence of strains depends upon the proportion of active cell infections which they produce and this proportion rises in a continuous series from that of the virus in carrier mice to that of ordinary MHV 1 and even to that of agents such as MHV 2 (mouse hepatitis virus described by Nelson, 1952) and MHV 3 (Dick *et al.* 1956) which are fully virulent in the absence of *E. coccoides*.

The author gratefully acknowledges the continued help of Dr Janet S. F. Niven, especially in connexion with experiments described herein and necessary preliminary experiments, and the useful criticism of Professor G. W. A. Dick, Dr F. B. Bang and Dr C. H. Andrewes, F.R.S. The author also wishes to thank Miss M. V. Mussett for checking the calculations and Mr A. W. Lane for technical assistance.

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## Quantitative Variations in the Bacterial Flora of Flatfish

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**SUMMARY:** Bacterial counts made, over a period of 27 months, on skin, gut and gill samples of freshly caught skate and lemon sole, using sea water-based and tap water-based media in parallel, revealed a seasonal variation in the size of the bacterial populations on the fish throughout the year. Evidence is presented for the view that the occurrence of maximum bacterial populations on fish is correlated with plankton outbursts. Sea water-based medium was generally superior to a tap water-based medium for isolating bacteria from fish; the importance of this as evidence of a specific marine bacterial flora is discussed.

Bacteria occur in appreciable numbers on the skin and gills and in the intestinal contents of fish (Harrison, Perry & Smith 1926). However, it is generally accepted that the internal organs and the muscle tissues of (healthy) live or freshly caught fish are sterile (Anderson, 1907; Proctor & Nickerson, 1935). The spoilage of fish as a foodstuff is due to the activities of bacteria normally present on the living fish which, after the animal's death, penetrate into the muscle tissues and decompose them. Consequently, the bacterial flora of different types of fish has been the subject of several investigations, the results of which were summarized by Shewan (1949). Most of these studies were confined to commercially important species of 'round' fish' such as cod, haddock and herring and have, in the main, consisted of qualitative analyses of the bacterial types isolated from them; flatfish such as skate, lemon sole, turbot, etc., have been little studied. However, the results of an investigation by Reay & Shewan (1949) into the numbers of bacteria on the skin of haddock at various times of the year indicated that there is probably a seasonal variation in the quantitative load of bacteria on this fish. This conclusion is not entirely unexpected since seasonal variations in the numbers of bacteria in sea water have been observed by workers in different parts of the world (Lloyd, 1930; ZoBell & McEwan, 1935; Wood, 1953) and sea water is undoubtedly the source of many of the bacteria found on fish.

Workers in the field of marine bacteriology have used a variety of media in investigations of the bacterial flora of sea water and of fish and this has made comparison of their results peculiarly difficult. Since it has been claimed that many bacteria of marine origin will not grow in the absence of sea water (ZoBell, 1946) the comparison of viable counts made in tap-water-containing and sea water-containing media is particularly difficult.

The investigation described below was carried out to determine whether seasonal variations occur in the bacterial flora of skate (*Raja* spp.) and lemon sole (*Pleuronectes microcephalus*) and to determine, so far as possible, what proportion of the bacteria on the fish require sea water in the media for their

growth, by comparing the results of viable counts obtained with sea-water media and fresh-water media. Skate and lemon sole were chosen as the subjects of this investigation for three reasons: (1) very little has been published concerning the bacterial flora of flatfish, the mode of life of which differs from that of the more free-swimming 'round' fish; (2) though they occupy the same environment and have a similar mode of life the two fish belong to different taxonomic groups; the skate is an elasmobranch while the lemon sole is a teleost; (3) both fish are readily obtainable in Aberdeen Bay all the year round.

#### METHODS

During the period October 1952 to December 1954 several freshly caught specimens of lemon sole and skate were examined each month. The fish were caught by trawl net some 10 miles off-shore in Aberdeen Bay. They were transferred from the net to sterile aluminium boxes with the minimum of handling and brought to the laboratory within a few hours of catching. During transit the boxes were surrounded with ice and the fish were frequently still alive on reaching the laboratory. On arrival they were killed when necessary, and skin samples of known uniform area and weighed gut and gill samples excised. Duplicate viable counts were carried out on the diluted samples using tap water-based media and sea-water-based media in the usual pour-plate technique.

Tap water-based medium (horse heart agar; HHA) contained 0.5 % sodium chloride, 1.0 % peptone and 1.5 % agar dissolved in a tap water extract of horse or ox heart (500 g. minced lean heart/1000 ml. water).

Sea water-based medium (sea water agar; SWA) contained 0.1 % Lab-Lemco, 0.1 % peptone, 0.1 % glucose, 0.005 % potassium phosphate and 1.5 % agar dissolved in sea water. The sea water was previously aged by storing it in a glass container in the dark for several weeks to eliminate the intrinsic bacteriostatic property of raw sea water (ZoBell, 1946).

Both media were sterilized by autoclaving at 15 lb./sq.in. (*c.* 121°) for 15 min. and the reaction adjusted to give a pH value after autoclaving of 7.6.

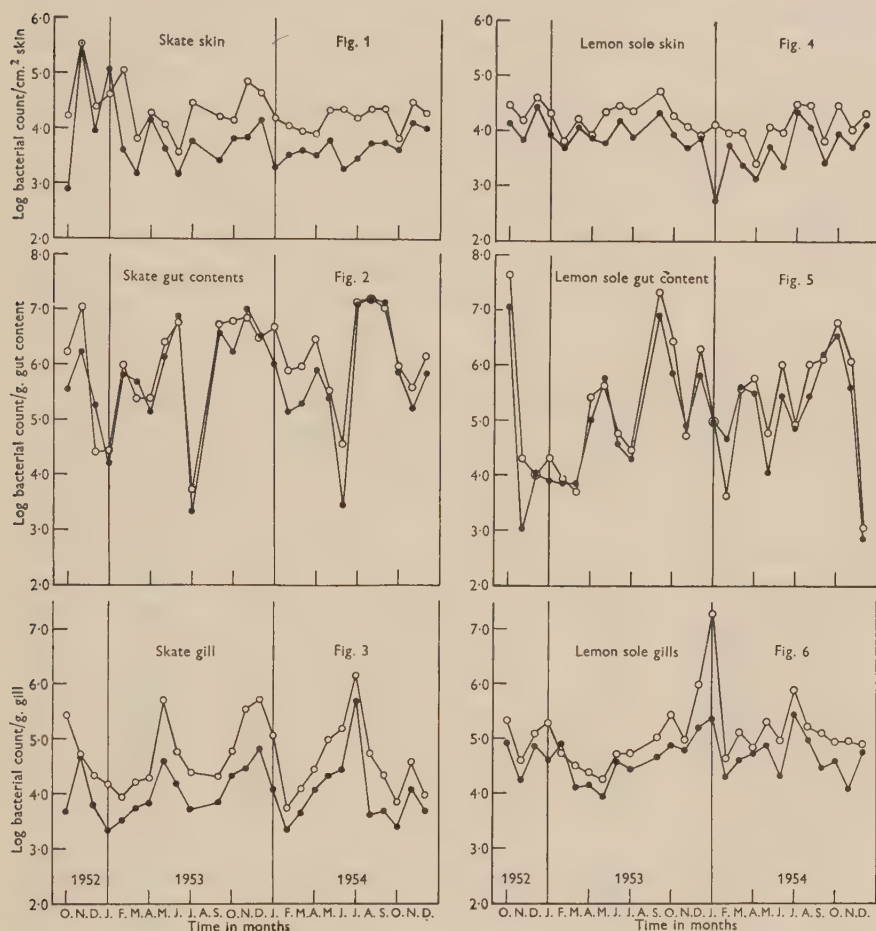
In each count, duplicate series of plates were incubated at 20° and 0° and the resulting colonies counted after 5 and 25 days, respectively. A few plates inoculated from the original suspension were incubated at 37° and counted after 3 days of incubation. These latter served primarily as a control of the counting technique since few organisms from fish caught in Northern waters will grow at temperatures greater than 30° (Schönberg, 1930).

#### RESULTS

The number of fish examined each month is shown in Table 1. The results of the counts carried out each month have been averaged and, for convenience of presentation (in view of the fluctuations from fish to fish), are recorded as the logarithm to the base ten of the number of bacteria/cm.<sup>2</sup> skin sample or/g. gut or gill samples.



Plates incubated at 37° rarely developed more than a few colonies and in no case did the count at this temperature exceed 3% of that at 20°. The values obtained for bacterial counts carried out at incubation temperatures of 0° and 20° are listed in Tables 2 and 3 and in Figs. 1-6 respectively.



Figs. 1-3. Bacterial counts (20°) in skate skin, gut contents and gills. Each point represents the average of one month's results —○—○—, counts using sea water agar (SWA); —●—●—, counts using tap water agar (HHA).

Figs. 4-6. Bacterial counts (20°) on Lemon sole skin, gut contents, gills. Each point represents the average of one month's results —○—○—, counts using SWA; —●—●—, counts using HHA.

In general it appears from the 20° counts that the numbers of viable bacteria on the skin of freshly caught skate and lemon sole vary between  $10^3$  and  $10^5$  organisms/cm<sup>2</sup>. On the gills the range is between  $10^3$  and  $10^6$  organisms/g. for skate and between  $10^4$  and  $10^6$  organisms/g. for lemon sole. The numbers of living bacteria in gut samples from both fish varied from  $10^3$  to  $10^7$  organisms/g.

Table 1. *Number of fish examined each month during period October (O.) 1952 to December (D.) 1954*

Year...	1952			1953											
	O.	N.	D.	J.	F.	M.	A.	M.	J.	J.	A.*	S.	O.	N.	D.
Skate	2	2	2	2	3	3	3	4	6	2	—	4	5	4	4
Lemon Sole	2	2	2	3	2	3	3	4	6	2	—	4	5	4	4
Monthly total	4	4	4	5	5	6	6	8	12	4	—	8	10	8	8

Year...	1954												Total samples
	J.	F.	M.	A.	M.	J.	J.	A.	S.	O.	N.	D.	
Skate	1	3	4	6	5	2	3	3	4	5	5	3	90
Lemon Sole	1	3	4	6	5	2	3	3	4	5	5	3	90
Monthly total	2	6	8	12	10	4	6	6	8	10	10	6	180

\* Trawler not operating.

Table 2. *Bacterial counts (0° incubation) from skate samples*

Month and year	Skin count on		Gut contents count on		Gills count on	
	HHA	SWA	HHA	SWA	HHA	SWA
	log count/cm. <sup>2</sup>		log count/g.		log count/g.	
Oct. 1952	2.86	4.15	5.25	4.26	4.10	5.16
Nov. 1952	4.82	4.83	4.28	2.48	4.19	3.38
Dec. 1952	3.78	4.11	4.01	3.76	3.53	4.07
Jan. 1953	4.07	4.19	4.31	4.64	3.14	3.87
Feb. 1953	3.42	3.84	5.37	5.24	3.55	3.75
Mar. 1953	3.05	2.90	4.93	4.84	3.55	3.87
Apr. 1953	4.16	4.12	5.56	4.73	4.01	3.92
May 1953	3.73	3.99	6.02	5.39	5.30	5.30
June 1953	3.24	3.52	6.83	7.06	4.26	4.57
July 1953	3.84	3.80	3.87	4.41	4.28	4.85
Aug. 1953	—	—	—	—	—	—
Sept. 1953	3.52	4.02	6.16	5.64	3.90	3.97
Oct. 1953	3.62	3.84	5.92	6.71	4.14	4.26
Nov. 1953	3.71	4.50	6.95	5.01	4.87	5.14
Dec. 1953	4.14	4.33	5.53	5.42	4.90	5.30
Jan. 1954	3.05	3.97	5.67	6.36	4.81	5.12
Feb. 1954	2.63	3.96	3.17	4.45	2.54	2.56
Mar. 1954	3.53	3.83	4.88	4.93	3.40	3.92
Apr. 1954	3.41	3.75	5.42	5.63	3.81	4.25
May 1954	3.61	4.23	5.22	5.36	4.21	4.84
June 1954	2.93	4.04	3.23	4.64	4.40	4.88
July 1954	3.22	4.18	5.95	6.16	4.83	5.36
Aug. 1954	3.22	4.19	7.14	7.11	3.44	4.70
Sept. 1954	3.13	4.09	5.82	6.70	2.94	3.04
Oct. 1954	2.79	3.55	5.78	5.74	2.88	3.71
Nov. 1954	3.26	4.20	4.98	5.23	3.69	4.43
Dec. 1954	3.26	3.85	5.56	5.40	3.16	3.63

HHA = horse heart agar; SWA = sea water agar.

During most months the count obtained by incubation at 0° was somewhat lower than that obtained at 20°, but the difference is not excessively great and in a few instances the count at 0° was actually higher than that at 20°.

With few exceptions the counts carried out on sea water agar (SWA) were higher than those obtained with horse heart agar (HHA) in the case of skin and gill samples (Figs. 7–12). Counts of gut samples showed little difference whether SWA or HHA was used.

Table 3. *Bacterial counts (0° incubation) from lemon sole samples*

Month and year	Skin count on		Gut contents count on		Gills count on	
	HHA	SWA	HHA	SWA	HHA	SWA
	log count 1 cm. <sup>2</sup>		log count/g.		log count/g.	
Oct. 1952	4.17	4.70	7.22	5.99	4.98	4.95
Nov. 1952	3.68	3.99	3.00	3.00	4.04	4.21
Dec. 1952	4.31	4.40	4.28	4.28	4.61	4.84
Jan. 1953	3.82	4.00	4.15	4.01	4.60	4.88
Feb. 1953	3.65	3.62	3.41	4.48	4.20	4.14
Mar. 1953	3.79	4.18	3.30	3.39	4.09	4.24
Apr. 1953	3.78	3.74	5.63	5.05	4.20	4.40
May 1953	3.41	4.12	5.72	5.55	4.21	4.21
June 1953	4.28	4.30	5.32	4.84	4.46	4.80
July 1953	3.34	4.75	2.60	5.50	4.34	4.68
Aug. 1953	—	—	—	—	—	—
Sept. 1953	4.08	4.27	6.25	6.31	4.32	4.79
Oct. 1953	3.89	4.20	5.56	5.91	4.75	5.07
Nov. 1953	3.65	3.85	4.20	4.04	4.33	4.50
Dec. 1953	3.57	3.81	4.73	4.64	5.13	5.67
Jan. 1954	3.59	4.03	2.00	2.00	5.92	6.60
Feb. 1954	3.36	3.64	4.16	3.22	4.27	4.43
Mar. 1954	3.44	3.80	5.72	5.68	4.72	5.05
Apr. 1954	2.94	3.29	5.42	5.79	4.95	5.37
May 1954	3.70	4.01	5.21	4.46	4.73	5.20
June 1954	3.38	3.90	5.59	5.80	4.52	4.74
July 1954	4.15	4.59	4.86	4.89	5.40	5.66
Aug. 1954	3.91	4.40	5.56	6.19	4.65	5.18
Sept. 1954	2.94	3.33	4.45	5.96	4.17	4.87
Oct. 1954	3.75	4.11	5.39	5.06	4.18	4.62
Nov. 1954	2.84	3.86	5.39	5.49	3.72	4.42
Dec. 1954	3.96	4.14	1.00	1.00	4.21	4.57

HHA=horse heart agar; SWA=sea water agar.

Figs. 1–6 show that there was some periodicity in the occurrence of high and low counts during the period of the investigation. Indications of a seasonal recurrence of high and low loads of bacteria on the fish examined are most apparent in the graphs showing the counts on gill samples. Counts at 20° and 0° showed similar fluctuations throughout the period of the experiment whether SWA or HHA was used as incubation medium. From the gill counts it appears that bacteria occur in greater number on skate in early summer and early winter, and on lemon sole in mid-winter and possibly again in summer. Unfortunately, owing to the non-availability of the fishing boat at the time, no

samples were obtained in the latter half of July or in August, 1953. This makes it difficult to assess the importance of the summer maximum which occurred quite clearly in the lemon sole gills count curve for 1954.

#### DISCUSSION

The size of the bacterial populations on the skin and gills and in the gut contents of both skate and lemon sole is within the limits quoted by Shewan (1949) for the normal bacterial load on fresh or living fish. Shewan's figures were derived principally from the results of investigations carried out on 'round' fish, so that it appears that in this respect there is little difference between the bacterial loads on 'round' fish and on flatfish. This finding is somewhat unexpected in view of the much greater populations of bacteria present in bottom deposits as compared with those occurring in the over-lying water (Waksman, 1934). Though it is true that many 'round' fish are demersal feeders, spending much of their time in proximity to the sea bed, they are not exposed to the same degree of contamination as the flatfish which lie directly on the bottom and frequently burrow into the sand or mud deposited there. It seems likely therefore that the similarity between the numbers of bacteria occurring on the two types of fish is due to the selective effect exerted on adventitious bacteria by the conditions of the environment provided by the exposed surfaces of the fish body.

In considering the changes in numbers of bacteria present on flatfish as revealed in this investigation, it is necessary to examine briefly the validity of the count values from the three sample sites as indices of seasonal trends. The value of gut sample counts in assessing the effect of season on the flora of fish is questionable since the bacteria present in the gut are a function of the food ingested rather than of any intrinsic property of the fish. Thus several workers have established that in non-feeding fish the gut is virtually sterile (see Margolis, 1953); this has been confirmed in the present investigation in the case of lemon sole, many of which do not feed during the December–February period. Though feeding habits are related to the spawning cycle in lemon sole and are therefore, to some extent, seasonal this is not so in the case of skate. Nevertheless, the curve of skate gut counts shows more evidence of periodicity than that of lemon sole and so it seems that such curves are not simply bacterial records of feeding habits. Possibly some physiological change in the fish, which may or may not be seasonal, is responsible. In any case the protected situation of the gut flora makes it somewhat insensitive to seasonal changes in the environment of the fish and for this reason alone it is unlikely to be a good indicator of overall seasonal effects.

Skin counts, while showing a tendency to fluctuate more or less regularly with season, do not present any very clear-cut picture. This is most probably due to the effect on the bacterial flora of the fish of the catching procedure, in the course of which the animals are swept along over the bed of the ocean and tumbled against each other and against rocks, ropes, etc. Such treatment must appreciably alter the numbers of bacteria on the skin of the fish, and for this



reason the results obtained from skin samples, while acceptable as providing an indication of the actual variation in the bacterial population of the living fish, cannot be accepted as accurately describing these variations.

The counts on gill samples are considered to represent most nearly the true state of the bacterial population on a living fish. The gills, fully exposed to the aqueous environment of the free-swimming fish, are largely protected from the effects of the trawling operation when the fish is caught and it seems probable that the numbers of viable bacteria present on the gills are much the same before and after capture. Thus the pattern of seasonal variation so clearly delineated in the curves derived from the gill counts is considered to represent the actual changes on the living fish fairly accurately.

The difference in the shapes of the corresponding gill counts curves for skate and lemon sole indicates that, despite the overall quantitative similarity in the bacterial populations of different types of fish, species is of some importance in determining the concomitant bacterial population.

The seasonal occurrence of large numbers of bacteria in the sea has been thought by some workers to be correlated with either maximum water temperatures (ZoBell & Feltham, 1934) or with plankton outbursts (Lloyd, 1930; ZoBell & McEwan, 1935). The temperature of a fish is dependent on that of the surrounding water so that, if temperature is the factor of greatest importance in determining the bacterial population of sea water, it is likely to be of similar importance in the case of fish. However, no coincidence of high water temperatures with high bacterial loads on fish was observed in this investigation and temperature does not, therefore, appear to constitute the main determinative factor in this respect. The seasonal outbursts of plankton are limited during their period of growth to the surface layers of the sea and it would seem unlikely on a first estimate that they would affect the bacterial flora of flatfish which live exclusively at the bottom of the sea. Nevertheless, it was observed that highest counts were obtained from fish caught some 2 months after the spring and autumn plankton outbursts. In Aberdeen Bay during the period of the investigations the outbursts occurred in March and September in 1953 and in early March and early September in 1954 (Dr J. H. Fraser, personal communication) and this can be seen to correlate with the incidence of maxima in Figs. 3 and 6 in the way described above.

After the peak of plankton growth has passed, the decaying remains of the constituent plants and animals fall slowly to the bottom of the sea where they provide an additional source of organic nutrients for the bacteria growing there and also add a new quota of bacteria to this population, consisting of the organisms associated with the decaying particles. It seems likely that in such conditions there would be an appreciable increase in the numbers of bacteria present in the bottom flora, and in particular an increase of those types of bacteria best adapted for the breakdown of organic material of marine origin. Such bacteria are, by their biochemical capabilities, extremely well suited for life on the surfaces of fish, where a plentiful supply of organic nutrients is abundantly available, and an increase in the size of bacterial population on fish would be expected to occur in association with an increase in their

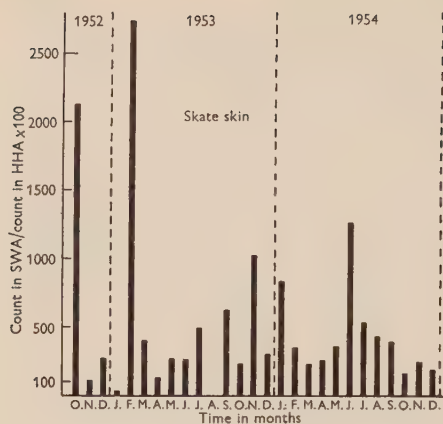


Fig. 7

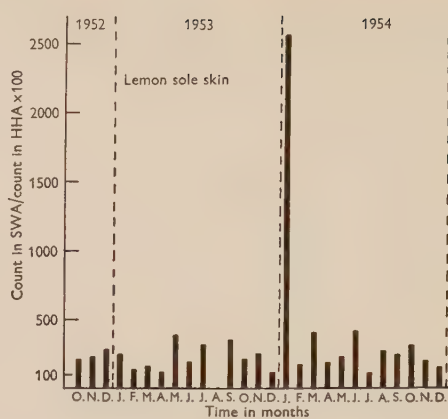


Fig. 10

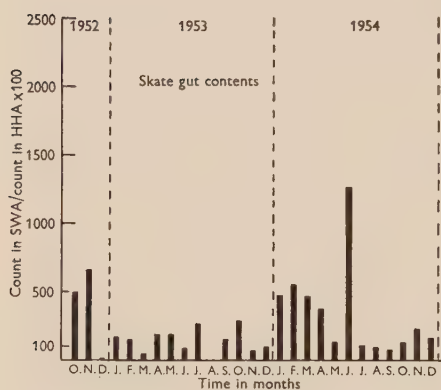


Fig. 8

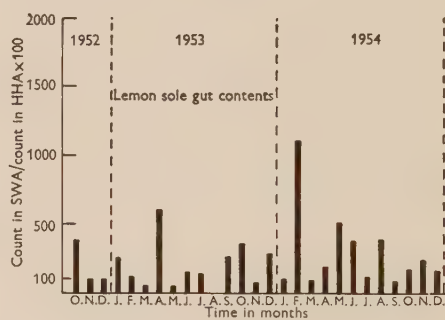


Fig. 11

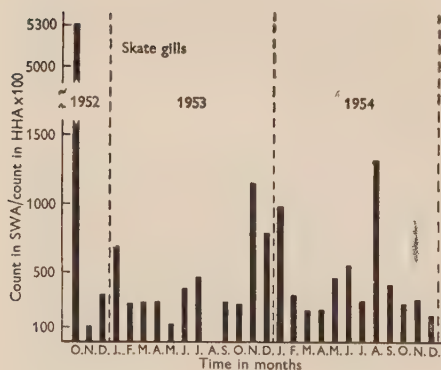


Fig. 9

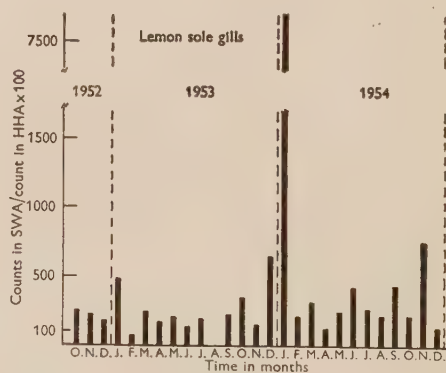


Fig. 12

Figs. 7-9. Skate skin, gut contents, gills, respectively. Count in sea water agar (SWA) as a percentage of count in horse heart agar (HHA). Incubation temperatures: 20°.

Figs. 10-12. Lemon sole skin, gut contents, gills, respectively. Count in sea water agar (SWA) as a percentage of count in horse-heart agar (HHA).

numbers, in the immediate environment. It seems probable therefore that the seasonal variation in the bacterial population of flatfish is determined, though indirectly, by the occurrence of plankton outbursts. Counts carried out at 20° and 0° showed practically identical variations during the course of the experiment, though the relative proportions of the flora appearing at the two temperatures were not constant. This indicates that the seasonal effect is probably exerted impartially on the total bacterial flora.

The high incidence of organisms growing at 0° among the bacteria from flatfish is in accord with the view that most fish bacteria are more or less psychrophilic (Shewan, 1949) and the very low counts obtained with 37° as incubation temperature confirm this viewpoint. It is probable, however, that the temperature relations of bacteria from fish are to some extent a function of the temperature of the sea water in which the fish live. Thus Wood (1953) recorded that a high proportion of the bacteria isolated from marine sources in warm Australian waters grow at 37°.

The results expressed as block diagrams in Figs. 7–12 represent the counts in SWA medium expressed as percentages of the corresponding counts in HHA medium. They demonstrate clearly the superiority of SWA as a medium for carrying out bacterial counts on fish except for counts on gut samples. In gut samples it was commonly found that the bacterial population consisted almost entirely of a culture of a single well-defined type of micro-organism which, though growing well in the presence of sea water, preferred a rather richer medium than SWA (Liston, 1954). Nevertheless, it appears from the skin and gill counts that there is a group of micro-organisms on fish, constituting a significant part of the total flora, which are sea water-loving or even nutritionally exacting for sea water. ZoBell and his co-workers have repeatedly claimed that there exists a specific marine bacterial flora (ZoBell & Upham, 1944; Zobell, 1946), the most definite characteristic of which is a requirement for sea water for growth on initial isolation. While most of the organisms isolated by the present writer from fish have proved to be able to grow on tap-water media either on initial isolation or after a primary culture on sea-water media, many certainly show a marked preference for the latter, both from the point of view of rapidity of growth and also quantity of growth. It is interesting that Wood (1953), though disinclined to support the hypothesis that a specific marine flora exists, recorded that bacterial counts on samples from 'truly oceanic stations', were four times as high on sea water agar as on fresh water agar. Thus it would appear that there is a bacterial flora in the sea, well adapted to the environmental conditions and in consequence more or less exacting, nutritionally, for sea water or some of the constituents of sea water (McLeod, Onofrey & Norris, 1954). The results described above indicate that the bacteria on fish belong to species encountered in the open sea. Whether the bacterial flora of the fish is a function of that of the surrounding sea water or vice versa it is not possible to decide at present; in view of the low nutrient content of sea water it is probable that the sea will play a passive role, acting merely as a vehicle of transport for bacteria derived from suitable sites of growth in the marine environment.

I wish to express my gratitude to Dr J. H. Fraser of the Marine Laboratory, Aberdeen, who provided me with information on plankton outbursts.

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## The Action of Phenol and 2-Phenoxyethanol on the Oxidation of Various Substances by *Escherichia coli* and by a Disrupted Cell Preparation of the Organism

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**SUMMARY:** The effects of phenol and 2-phenoxyethanol on the oxygen uptake of washed suspensions and a disrupted cell preparation of *Escherichia coli*, with different substrates, has been further investigated. Stimulation of oxygen uptake with mannitol or glucose as substrate was not due to an increase in the viable population or associated with an uncoupling effect. The results obtained with a disrupted cell preparation capable of oxidizing glucose and lactic acid to pyruvic acid, on comparison with results with intact organisms, suggest that enzyme location may account for the differences in inhibitory activity obtained with different substrates.

Hugo & Street (1952) showed that the action of phenol and 2-phenoxyethanol (phenoxetol) on the course of oxygen uptake by washed suspensions of *Escherichia coli* differed markedly with different substrates. In the presence of phenol (0.1-0.2 %, w/v; 0.010-0.021 M) and 2-phenoxyethanol (0.1-0.2 %, w/v; 0.007-0.014 M) a stimulation of the rate of oxygen uptake of the order of 10-20 % occurred when glucose, mannitol or lactose was used as substrate; these same doses inhibited the rate of oxygen uptake by 10-15 % when succinate, lactate, pyruvate or acetate was the substrate. These results were difficult to explain on the hypothesis that these two antiseptics are non-specific protoplasmic poisons. Three working hypotheses were considered in an attempt to explain these effects of substrate on the response of oxygen uptake to the antiseptics: (i) that the differences reflect changes in the population of viable organisms; (ii) that the stimulations of oxygen uptake were associated with an uncoupling effect such as is shown by the nitrophenols; (iii) that the differences could be attributed to an effect of the antiseptic on permeability of the organisms to substrate, coupled with differences in the location of the enzymes involved in the oxidation of different substrates. The experiments now described were undertaken to evaluate these hypotheses.

### METHODS

**Chemicals.** Diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) were samples supplied by Messrs L. Light and Co. Ltd.; the former when assayed by the method of Racker (1950) was found to contain 82 %, w/w, DPN. Sugars and mannitol were of bacteriological grade as supplied by Messrs T. Kerfoot Ltd. The 2-phenoxyethanol was a sample which complied with the requirements of the British Pharmaceutical Codex. The methylene blue was a

sample from Messrs Hopkins and Williams Ltd.; all other chemicals were of A.R. quality. The 2:4-dinitrophenol (DNP) was recrystallized twice from ethanol. Lactic acid was neutralized to pH 7.0 with sodium hydroxide; sodium pyruvate was prepared by the method of Robertson (1942). Phosphate buffers were prepared from  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  and the pH value checked electrometrically. Ringer's solution (quarter strength) was prepared from solution tablets (Messrs Oxo Ltd.).

*Growth of organisms.* *Escherichia coli* (NCTC 5934) was grown for 48 hr. on a tryptic digest agar (125 ml.) contained in a 1 l. Roux flask, harvested in quarter-strength Ringer's solution, washed twice with this solution and standardized nephelometrically to contain 6.4 mg. dry wt. organism/ml.

*Oxygen uptakes* were determined by the direct method of Warburg. The gas phase was air and measurements were made at 37° with a shaking rate of 100 oscillations/min. Unless otherwise stated, the main compartment of the Warburg vessel contained 1 ml. of substrate (0.02 M) in phosphate buffer (0.05 M, pH 7.0), and 0.5 ml. of antiseptic solution or DNP; the side arm contained 0.5 ml. of standardized bacterial suspension; the centre well 0.2 ml., 20 % KOH, and folded filter-paper. 2-Phenoxyethanol is soluble in 43 parts of water at 15.5°. This fact necessitated a change in the above scheme when studying higher concentrations, which is described in the appropriate section.

*Viable counts* were made by serial dilution in quarter-strength Ringer's solution and plating on tryptic digest agar.

*Disruption of the bacterial cells.* Bacteria (2.5 g. wet weight), harvested from slopes in Roux bottles, were placed in the containers of the Mickle apparatus together with 3 ml. water and 5 g. of 1 mm. diameter 'Ballotini' beads, and were well chilled in melting ice. The tubes were then vibrated for 40 min., re-chilled and vibrated for a further 40 min. Almost complete disruption of the bacteria was achieved, as judged by the inspection of stained preparations. The contents of the tubes were transferred to a centrifuge tube by using 2 × 1.5 ml. of water and spun at 3000 r.p.m. for 30 min. The turbid supernatant fluid, c. 5 ml., was removed from the deposit of 'Ballotini' beads and larger cell fragments by means of a pipette and re-centrifuged at 19,000 r.p.m. in a refrigerated centrifuge (Measuring and Scientific Equipment Ltd.). This resulted in a yellow supernatant fluid and a light flocculent deposit. This yellow supernatant fluid contained no living organisms, but on examination in a bright light was seen to be slightly opalescent.

## RESULTS

### *Oxygen uptake and viability*

Viable counts were performed on the contents of the Warburg flasks after completion of the oxygen uptake experiments. The changes in counts and oxygen uptakes expressed as a percentage of the control are compared in Table 1, in which it can be seen that although there was a slight decrease in count there was no relationship between decrease in count and oxygen uptake. By serially diluting bacterial suspensions it was shown that, in the absence of antiseptics, oxygen uptake was directly proportional to the viable cell content of the

suspensions over the range corresponding to 3.2–0.32 mg. dry wt. bacteria/flask, so that oxygen uptake paralleled viable count and a decrease in number of viable organisms would result in a proportionate decrease in the oxygen uptake rate. The lack of correspondence between viable counts and oxygen uptake in the presence of antiseptic at low concentrations showed that the oxygen uptake responses were being determined by some other factor(s).

Table 1. *The effect of 0.1 % phenol and 0.2 % 2-phenoxyethanol on the viable count and rate of oxygen uptake  $\mu\text{l./min.}$  of Escherichia coli at 37°*

Substrate	0.1 % phenol		0.2 % 2-phenoxyethanol	
	Viability, % of control without phenol	Rate of O <sub>2</sub> uptake, % of control without phenol	Viability, % of control without 2-phenoxyethanol	Rate of O <sub>2</sub> uptake, % of control without 2-phenoxyethanol
Mannitol	93	121.6	96.7	128.2
Glucose	94.2	110.3	94	108
Glycerol	99.4	96.5	100.2	93.5
Lactate	96	73.3	95.7	65
Succinate	95.8	45.3	95.3	61.4

At phenol concentrations of 0.75–1.0 %, w/v (0.079–0.106 M) and 2-phenoxyethanol concentrations of 1.0–1.2 %, w/v (0.072–0.086 M) a slow but measurable oxygen uptake on lactate in the first case and on lactate or succinate in the second case occurred, despite the fact that these high antiseptic concentrations sterilized the contents of the Warburg flask in 30 min. These same concentrations caused an abrupt and complete cessation of oxygen uptake with the following substrates: mannitol, glucose, lactose, pyruvate, glycerol.

*Comparison of the behaviour of phenol, 2-phenoxyethanol and 2:4-dinitrophenol*

Nitrophenols are known to stimulate the rate of respiration of bacteria (Shoupe & Kimler, 1934) and to cause the complete oxidation of substrates in certain cases by inhibiting assimilation (Clifton, 1937). In the experiments reported above and in earlier work (Hugo & Street, 1952) the concentration of substrate was maintained in excess so that depletion of substrate would not occur. In the present experiments it was necessary to investigate complete oxidation and hence the concentrations of substrate were adjusted to ensure that the total volume of oxygen required could be measured on the manometer scale. Mannitol and glucose at  $2.5 \times 10^{-3}$  M and lactate at  $5 \times 10^{-3}$  M were used as substrates. Otherwise the conditions were as stated under methods and as used in previous experiments (Hugo & Street, 1952). The concentrations of inhibitors were: phenol 0.1 % w/v (0.010 M), 2-phenoxyethanol 0.2 % w/v (0.014 M), 2:4-dinitrophenol  $2.5 \times 10^{-4}$  M.

The results are shown in Table 2, where it can be seen that neither phenol nor 2-phenoxyethanol (at concentrations stimulatory to the rate of oxygen uptake) significantly increased the total oxygen uptake. In contrast, 2:4-dinitrophenol caused an increase in the total oxygen consumption. This,



however, did not reach the theoretical value for complete oxidation; other reports record the complete oxidation of substrates by *Escherichia coli* in the presence of this concentration of the nitrophenol (Clifton, 1937).

Table 2. *Comparison of the effect of phenol, 2-phenoxyethanol and 2:4-dinitrophenol on the oxidation of mannitol, glucose and lactate by washed suspensions of Escherichia coli*

Substrate	Expt.	Control	With 0.010 M- phenol	With 0.014 M- phenoxetol	With $2.5 \times 10^{-4}$ 2:4- dinitrophenol	Theoretical oxygen uptake for complete oxidation, $\mu$ l.
Total oxygen uptake, $\mu$ l.						
Mannitol, $2.5 \times 10^{-3}$ M, 1.0 ml.	1	217	215	219	294	364
	2	222	213	217	286	
Glucose, $2.5 \times 10^{-3}$ M, 1.0 ml.	1	230	224	219	277	336
	2	237	243	231	283	
Lactate, $5.0 \times 10^{-3}$ M, 1.0 ml.	1	214	227	209	291	336
	2	224	218	228	287	

Fig. 1 also illustrates the difference in behaviour of phenol and 2:4-dinitrophenol. With mannitol or glucose as substrates all three compounds stimulated the rate of oxygen uptake but only the dinitrophenol affected the total oxygen consumption. With lactate as substrate the rate of oxygen uptake was not stimulated by any of the three compounds, though with dinitrophenol the total oxygen uptake was increased.

#### *Permeability and enzyme location*

Information concerning the role of permeability was sought by comparing the activities of intact and disrupted organisms and the action of antiseptics upon these two systems. Oxygen uptakes of the order of  $60 \mu$ l./hr. occurred when the disrupted organism preparation was incubated with lactate as substrate, and the production of pyruvic acid was demonstrated in the pooled contents of five Warburg flasks by paper chromatography of the 2:4-dinitrophenylhydrazones according to the method of Cavallini, Frontalli & Toschi (1949). Pyruvate itself was not oxidized by the preparation.

The dry weight of the disrupted organism preparations lay between 14 and 18 mg./ml. The  $Q_{O_2}$  value for lactate ranged between 3.3 and 4.3. The average  $Q_{O_2}$  value with the intact organism was 88 and thus the activity of the disrupted organism was of the order of 1/20 to 1/27 that of intact organisms.

The preparation was without action on glucose. However, addition of DPN, ATP, MB and  $Mg^{++}$  showed that addition of DPN caused a significant oxygen uptake in the presence of glucose (Table 3) and led to the production of pyruvic acid. It will also be observed that the oxygen uptake was greater in the absence of glucose (flask 12) than in the absence of DPN (flask B) and it is possible that



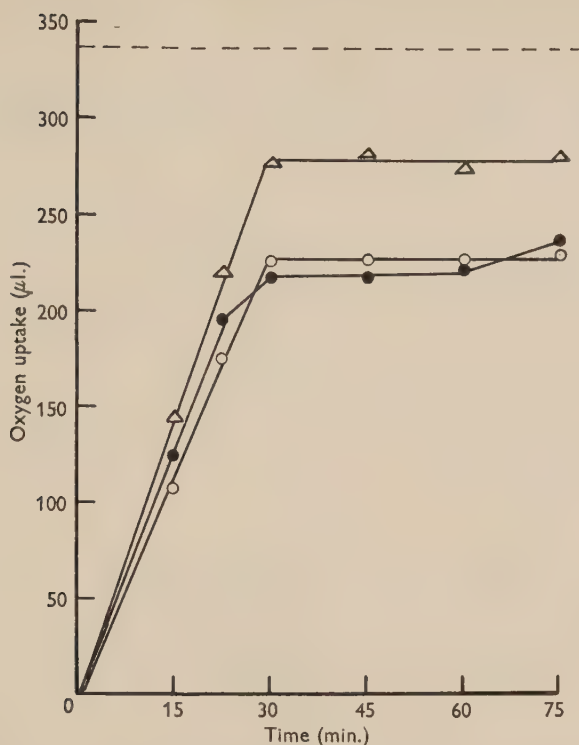


Fig. 1. Comparison of the action of phenol ( $0.010M$ ), ●—●, and 2:4-dinitrophenol ( $2.5 \times 10^{-4}M$ ), Δ—Δ, on the oxidation of 1.0 ml. of  $2.5 \times 10^{-2}M$ -glucose by 0.5 ml. of a washed suspension of *Escherichia coli* containing 6.4 mg. dry wt. bacteria/ml. ○—○, uptake in the absence of an inhibitor; ----, uptake level for complete oxidation.

Table 3. *The effect of cofactors and methylene blue on the oxidation of glucose by a disrupted bacterial preparation from Escherichia coli*

	Flask identification						
	10	E	12	B	9A	1	2
Side arm							
0.5 ml. of disrupted bacterial preparation	+	+	+	+	+	+	0
Main vessel							
0.5 ml. 0.08 M-methylene blue	+	+	+	+	+	0	+
0.5 ml. 0.01 M-ATP	+	+	+	+	0	+	+
0.5 ml. 0.004 M-DPN	+	+	+	0	+	+	+
0.5 ml. 0.001 M-MgCl <sub>2</sub> . 6H <sub>2</sub> O	+	0	+	+	+	+	+
0.5 ml. 0.04 M-glucose in 0.1 M-phosphate buffer pH 7.0	+	+	0	+	+	+	+
Expt. 1. O <sub>2</sub> uptake μl. after 100 min.	254	252	103	93	249	312	3
O <sub>2</sub> uptake corrected for uptake without substrate (103 μl.)	151	149	0	-10	146	209	0
Expt. 2. O <sub>2</sub> uptake μl. after 120 min.	210	218	67	37	204	243	0
O <sub>2</sub> uptake corrected for uptake without substrate (67 μl.)	143	151	0	-30	137	176	0

in flask 12 some component of the disrupted organism preparation was being oxidized, this reaction requiring DPN.

The  $Q_{O_2}$  value for glucose calculated from the data in Table 3 flask 1 was 6.9, 1st experiment and 5.8, 2nd experiment. The average  $Q_{O_2}$  value for the intact organism was 100, thus the activity of the disrupted organism was of the order of 1/17 to 1/14 of the intact.

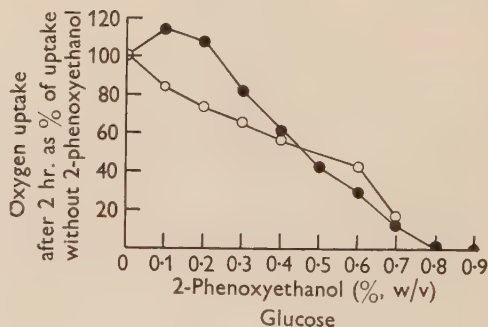
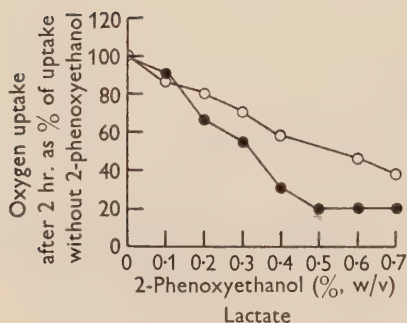
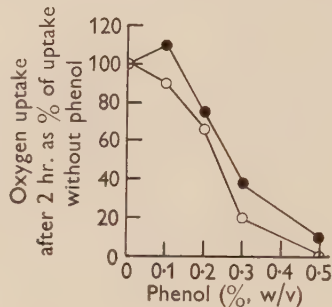
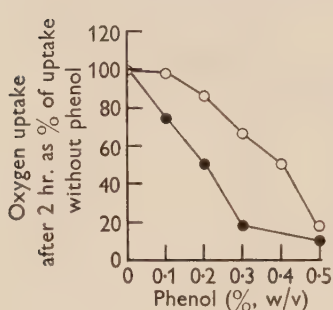


Fig. 2

Fig. 3

Fig. 2. Comparison of the action of phenol and 2-phenoxyethanol on the oxidation of lactate by intact cells, ●—●, and a disrupted cell preparation, ○—○, of *Escherichia coli*. Warburg flasks filled as follows: main compartment; 1.0 ml. 0.02 M-lactate in 0.05 M-phosphate buffer (pH 7.0), 0.5 ml. water or antiseptic solution. Side arm: 0.5 ml. intact cells or disrupted organism preparation. Centre well: 0.2 ml. 20 % KOH and filter-paper. 2-phenoxyethanol (0.8–1.0 %, w/v; 0.06–0.07 M). Main compartment: 0.5 ml. of 0.4 M-lactate in 0.1 M-buffer and 1.0 ml. of antiseptic solution. Duration of experiment 2 hr.

Fig. 3. Comparison of the action of phenol and 2-phenoxyethanol on the oxidation of glucose by intact cells, ●—●, and a disrupted cell preparation, ○—○, of *Escherichia coli*. Warburg flask filled as follows: Main compartment: 0.5 ml. 0.04 M-glucose in 0.1 M-phosphate buffer (pH 7.0), 0.5 ml. 0.004 M-DPN, 0.5 ml. water or antiseptic solution. Side arm and centre well as in Fig. 2. 2-phenoxyethanol (0.8 and 1.0 %, w/v; 0.06–0.07 M). Main compartment: 0.5 ml. 0.04 M-glucose and 0.004 M-DPN in 0.1 M-buffer, 1 ml. antiseptic solution. Duration of experiment 2 hr.

Unlike the oxidation of lactate, the rate of oxidation of glucose often decreased during the course of the experiment. This decrease was traced to the presence of a DPN-destroying system in the disrupted organism preparation, as indicated by incubating the disrupted organism preparation with DPN for

6 hr. at 37°. On subsequent addition of glucose in phosphate buffer no oxygen uptake could be detected, nevertheless on the addition of fresh DPN, oxygen uptake occurred.

The action of phenol and 2-phenoxyethanol on the oxidation of lactate and of glucose by intact organisms and by the disrupted organism preparation was now compared.

*Lactate oxidation.* A typical experiment comparing results with a disrupted bacterial preparation and intact organisms is shown in Fig. 2. When lactate was supplied as substrate the disrupted preparation appeared less susceptible than the intact cell, but the difference in the  $Q_{O_2}$  values of the intact and disrupted organism should be borne in mind.

*Glucose oxidation.* Results, with a disrupted bacterial preparation are compared in Fig. 3 with results observed with intact cells. Stimulation of oxygen uptake with glucose was not obtained with the disrupted preparation.

### DISCUSSION

The results of the first two groups of experiments supported the view that the stimulation of oxygen uptake by phenol and 2-phenoxyethanol did not result from changes in the viable count or from an uncoupling effect such as is known to be the cause of the enhanced respiration occasioned by nitrophenols. Attention was therefore turned to the possibility that the antiseptics caused an increase in the permeability of the bacteria, thereby facilitating substrate oxidation. This led to the conclusion that the stimulation of oxygen uptake obtained with low concentrations of phenol and 2-phenoxyethanol in the presence of glucose, mannitol or lactose is explicable on the hypothesis that the enzyme or enzymes responsible for their oxidation are located within rather than at the surface of the organism, and that the first effect of the antiseptic is to effect a general increase in the permeability. Such an explanation would support the finding of Deere (1939) and of Gale & Taylor (1947) that phenol was able to effect a general increase in the permeability of bacterial cells. The inhibition of oxygen uptake observed with other substrates at similar low concentrations of phenol or 2-phenoxyethanol would then presuppose that the enzymes concerned in these cases were at or near the surface of the organism and associated with centres at which the phenols were adsorbed. The glucose oxidizing system in the disrupted bacterial preparation studied in the present experiments showed no stimulation when treated with concentrations of the antiseptics which would have caused stimulation of the oxygen uptake of intact organisms. This supports the hypothesis that increased permeability resulting from the action of the anti-bacterial agent is the operative factor causing respiratory stimulation with those substrates; under the conditions of these experiments the rate of oxidation of certain substrates is probably limited by the rate of diffusion across a permeability barrier separating enzyme and substrate and this rate is increased by the antiseptic.

The action of CTAB (cetyltrimethylammonium bromide) calls for some comment. It has been shown by several workers (Gale & Taylor, 1947; Salton,

1950, 1951) that it causes an increase in permeability resulting in the leakage of cellular nitrogen and phosphorus into the external medium. In this respect its action is similar to that of phenol (Gale & Taylor, 1947). It might therefore be expected that CTAB at low concentrations would cause the acceleration of oxygen uptake with the same group of substrates as phenol. Such respiratory stimulation was not detected in the experiments reported by Hugo & Street (1952) nor in the work of Baker, Harrison & Miller (1940) who examined a range of quarternary ammonium compounds. The large size of the CTAB cation and its net positive charge are both properties which distinguish it from phenol and 2-phenoxyethanol and the strong attraction of the cation for the negatively charged bacterial surface, although initiating outward diffusion of cellular components, may also retard the entry of substrates into the bacterial cell or their metabolism at the cell surface, thereby accounting for the inhibiting effect independent of substrate. Ample evidence for the strong adsorption of cationic antiseptics by Gram-negative bacteria has been obtained by electrophoretic studies (Dyar & Ordal, 1946; McQuillen, 1950).

The production of pyruvic acid from glucose by the preparations of disrupted organisms is of interest. The present preparations resemble in some ways those prepared from *Escherichia coli* by Still (1940). Still's preparations converted hexosediphosphate to phosphoglyceric acid and required DPN for activity. It also seems that Still's preparation contained enolase, as sodium fluoride was included in the preparation 'to prevent formation of phosphopyruvic acid from phosphoglyceric acid'. In fermentation the DPN is reduced as a result of the oxidation of glyceraldehyde phosphate to diphosphoglyceric acid and is then re-oxidized by the simultaneous reduction of acetaldehyde, pyruvic acid or possibly other organic compounds with production of ethanol or lactic acid. Reduced DPN can however react with molecular oxygen provided a flavoprotein enzyme and one or more of the cytochrome pigments are present. There is thus presumptive evidence that the preparations used in the present investigation and those obtained by Still contained the components necessary for the reduction of molecular oxygen. Stimulation of oxygen uptake by low concentrations of phenol or 2-phenoxyethanol did not occur with intact organisms when oxidizing pyruvate. Thus the stimulated reaction must be one or more of those responsible for the conversion of glucose to pyruvate, and although this reaction is also carried out by the disrupted organism preparation stimulation did not occur. The pyruvate oxidizing system of the intact organisms is apparently not present in the disrupted cell preparation, or is inactivated or destroyed.

The oxidation of lactic acid proceeded without addition of cofactors and pyruvic acid was produced. Still (1941), with *Escherichia coli*, found that his cell-free preparations required methylene blue for activity with molecular oxygen. The activity of the preparations used in the present investigation was not increased in the presence of methylene blue. Knox, Auerbach, Zarvdnaya & Spirtes (1949) obtained an extract from a strain of *E. coli*, by grinding in the Booth-Green mill, which was able to react directly with molecular oxygen.



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## Loss of Group and Type Reactions by Tetracycline-resistant *Streptococcus pyogenes*

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**SUMMARY:** Four strains of *Streptococcus pyogenes* were found to have lost their group and type serological reactions after acquiring resistance to tetracycline antibiotics *in vitro*. These degraded variants were non-haemolytic and weakly fibrinolytic; they failed to grow in fresh human blood. Some of the degraded strains were found to have acquired the property of fermenting raffinose. Antisera prepared against three degraded strains revealed some antigenic relationship between degraded forms derived from different parent strains, and between degraded and parent organisms.

A variant of *Streptococcus pyogenes* which was resistant to the tetracyclines and would not grow aerobically on ordinary culture media has been isolated from a number of burns in this hospital and described elsewhere (Lowbury & Hurst, 1956). We have tried without success to obtain these 'AN' forms *in vitro* by cultivating typical *S. pyogenes* in media containing tetracyclines. In the course of these studies, however, we found another atypical form of *S. pyogenes* which showed increased resistance to the tetracyclines and a loss of both group and type antigens. We record here some studies on these degraded variants.

### *Strains of Streptococcus pyogenes used*

The three strains (328, 924 and 7473) which were used in most of these experiments were aerobic tetracycline-sensitive organisms isolated from burns which later yielded tetracycline-resistant anaerobic (AN) forms of *S. pyogenes*. One other strain (6357) from a burn was also used in some of the experiments.

### *Production of degraded strains*

**Methods.** To obtain streptococci resistant to the tetracyclines, broth cultures of strains 328 (type 5/12/54), 924 (type 5/44) and 7473 (type 4) were subcultured by transferring one drop to each of a series of doubling dilutions of tetracycline and of oxytetracycline, separately, in nutrient broth. The highest concentration showing growth after 24 hr. at 37° was subcultured to tubes containing that concentration and also twice and half that concentration of the antibiotic in broth. Subcultures were made in this way daily, the concentration of antibiotic being increased as the strains acquired resistance. Cultures were set up in duplicate, one set being incubated aerobically and the other anaerobically at 37° overnight. They were subcultured on horse-blood agar, also in duplicate for aerobic and anaerobic incubation.

As an alternative method, strains 328, 924 and 6357 were inoculated in antibiotic broth as described above, and subcultured to blood agar; from blood-

agar subcultures non-haemolytic colonies, when present, were picked to further antibiotic broth, the concentration of antibiotic being increased as the organisms acquired resistance.

Sensitivity to tetracycline and to oxytetracycline was assessed as the minimal inhibitory concentration recorded on each subculture.

Haemolysis of blood agar was recorded at each subculture, and tests for soluble haemolysin were made at the end of the series of subcultures by a standard method (see Mackie & McCartney, 1953).

Late subcultures in the first experiment and all subcultures in the second were tested for serological group by Lancefield's method; many subcultures were also tested by Fuller's method.

Morphology was examined in Gram-stained and in nigrosin films of the original cultures and of the final subcultures.

*Results.* After 22 subcultures in the first experiment all the strains (3 from tetracycline and 3 from oxytetracycline) showed increased resistance to the antibiotics, the minimal inhibitory concentrations of both antibiotics rising about thirty-fold (from 0.2–0.4 to 6–12  $\mu\text{g./ml.}$ ). The final subcultures failed to haemolyse blood agar or to show a soluble haemolysin; all of them had lost their group reaction but retained characteristic streptococcal morphology. Subcultures as late as the 19th, which had acquired some resistance to tetracycline, still showed the presence of group A reaction. Some of the final subcultures showed a minute colony form. All of them grew as well on aerobic as on anaerobic culture.

In the second experiment, strains 328 and 6357, but not strain 924, yielded non-groupable, tetracycline-resistant variants which appeared within 7 days. This method (picking non-haemolytic colonies to antibiotic broth) appeared to be more reliable as well as quicker than serial subculture through antibiotic broth which, in several later experiments, failed to yield degraded streptococci.

#### *Other features of the degraded streptococci*

*Fermentation reactions.* Degraded forms and their parent strains were tested for fermentation of glucose, lactose, galactose, maltose, sucrose, fructose, mannitol, sorbitol, raffinose, inulin and salicin in Hiss's serum water incubated aerobically at 37°. Tests were examined after 18 hr. and again after 48 hr. of incubation. The parent strains fermented all the sugars except mannitol, sorbitol, raffinose and inulin. The degraded strains fermented the same sugars, but five out of six of them also fermented raffinose.

*Tests for fibrinolysin.* The parent organisms and degraded forms of strains 328 and 6357 were tested for fibrinolysin (streptokinase) production in the manner described by Tillett & Garner (1933). The original strains caused complete lysis of the fibrin clot within 25 min. and 35 min. respectively; in this time no lysis was produced by any of the degraded strains, but partial lysis by all of them was detected after 2 hr.

*Bacitracin sensitivity.* Maxted (1954) showed that streptococci of group A were almost always sensitive, and streptococci of other groups almost always resistant to bacitracin. We tested the bacitracin-sensitivity of our



original strains and of the degraded variants by a ditch plate method, with 10 units bacitracin/ml. in the agar ditch. There was no difference in the width of the zones of inhibition shown by the degraded forms and sensitive controls (e.g. the parent strains of *Streptococcus pyogenes*) inoculated on the same plate.

*Mouse virulence.* As the parent strains were found to be avirulent on intra-peritoneal and intracerebral injection into mice, no tests were made with the degraded variants.

*Growth in heparinized human blood.* Tubes containing 1.0 ml. fresh heparinized human blood were inoculated each with one drop of the degraded forms of strains 328 and 6357, grown overnight in nutrient broth, and diluted  $10^{-3}$  and  $10^{-5}$ . A similar series of inoculations in fresh blood were made with the parent streptococci. Broth tubes were inoculated as controls. After 24 hr. at  $37^{\circ}$  the tubes were examined and subcultured to blood agar. A heavy growth of the parent streptococci was found in subcultures from incubated blood, but there was no growth from either of the degraded forms after incubation in blood. All the broth controls showed a heavy growth of streptococci. The results of this experiment suggest that the degraded forms would have diminished virulence for man.

*Serological typing.* Parent and degraded cultures of strains 328, 924 and 7473 were typed for us by Dr W. H. H. Jebb of the Oxford Public Health Laboratory, and some also by Dr R. E. O. Williams at the Streptococcal Reference Laboratory, Central Public Health Laboratory, Colindale. All of the degraded strains were found to be non-typable by agglutination and precipitation methods.

*Immunization of rabbits with degraded streptococci.* Antisera were prepared against degraded strains 328, 914 and 7473 by intravenous inoculation of trypsinized broth cultures resuspended to 1/10 of the original volume in physiological saline; 1.0 ml. of the suspension was injected on 3 consecutive days each week over a period of 4 weeks. Trypsinized vaccines were used, as recommended by McCarty & Lancefield (1955), to facilitate the production of antisera. The rabbits were bled for serum 10 days after the last injection. Slide-agglutination tests were made with doubling dilutions (1/5 to 1/160) of these sera and of sera from unimmunized rabbits on all the degraded and all the parent streptococci. Absorption of the antisera was not attempted.

The results (see Table 1) show that the agglutinins which appeared acted not only against the immunizing strains, but also against some of the other degraded strains; two of the parent streptococci were agglutinated by one of the antisera (7473), but the other antisera did not agglutinate either the homologous or the heterologous parent strains. An unrelated strain of *Streptococcus pyogenes* (293) selected at random from cultures of burns was not agglutinated by any of the antisera.

*Morphology.* Degraded strains obtained by the first method were Gram-positive cocci in long chains and were indistinguishable in morphology from the parent streptococci. By the second method the degraded organisms appeared as short chains of very small Gram-positive cocci. These differences in morphology persisted on subculture of each of the strains.



*Reversion to characteristics of the parent strain.* After 50 daily subcultures in broth, two of the degraded strains (both derived from 328) were non-haemolytic and could not be grouped by Fuller's or Lancefield's methods. Similar results were obtained with shorter series of subcultures on degraded strains 924 (20 subcultures), 7473 (20 subcultures) and 6357 (30 subcultures). One culture of a degraded form of 924 which had been preserved for several weeks on Dorset egg medium was found to be giving a group A reaction and to grow in fresh heparinized human blood. For this isolated observation confirmatory material was, unfortunately, not available.

Table 1. *Serology of strains of Streptococcus pyogenes degraded after growth with tetracyclines*

Strains of <i>Streptococcus pyogenes</i>			Slide agglutination titre, for degraded streptococci				
			Of antisera to degraded strains			Of control sera*	
			328 c	924 b	7473 c	1	2
328 (a)	Parent	—	0	0	80	0	0
328 (b)	Degraded	Oxytetracycline	0	80	80	0	0
328 (c)	Degraded	Oxytetracycline	40	0	5	0	0
924 (a)	Parent	—	0	0	0	0	0
924 (b)	Degraded	Oxytetracycline	5	20	40	0	0
924 (c)	Degraded	Tetracycline	0	0	0	0	0
7473 (a)	Parent	—	0	0	5	0	0
7473 (b)	Degraded	Oxytetracycline	10	160	80	0	0
7473 (c)	Degraded	Tetracycline	10	160	160	0	0
293	Control	—	0	0	0	0	0

\* Sera from unimmunized rabbits.

## DISCUSSION

The ease with which streptococci of group A were found to lose their group and type reactions as well as their haemolytic properties in the presence of tetracyclines suggests a mechanism by which these organisms may escape detection and possibly persist in the host tissues after apparently successful chemotherapy. The altered forms, however, had probably lost virulence, as they failed to grow in fresh human blood and produced little fibrinolysin; moreover, they did not revert to the typical form after 20–50 subcultures in the absence of tetracyclines. The good results on skin grafting of burns after elimination of *Streptococcus pyogenes* by a tetracycline (Lowbury & Cason, 1954) suggest that degraded streptococci, if they are present in burns after a course of the antibiotic, do not exert any local pathogenic effect. The possibility of a reversion to virulent forms cannot, however, be excluded and is suggested by an isolated observation reported above.

Gezon (1948) reported acquired resistance in streptococci of group A grown in the presence of penicillin. Such strains also showed, in some instances, a loss of group antigen, and a loss of mouse virulence was always found. Sensitivity was restored by serial subculture on penicillin-free medium, and virulence was

occasionally restored by passage in normal mice. Similar results were reported with strains grown in the presence of chlortetracycline (Gezon & Fasan, 1950). The phenomenon described by these workers is probably the same as that observed by us, and it is interesting that it can be induced by different and unrelated antibiotics.

The antigenic change associated with acquired resistance to tetracyclines in our experiments contrasts with the persistence of group and type antigens and of virulence in the tetracycline-resistant 'AN' forms isolated from burns (Lowbury & Hurst, 1956). It differs, too, from the changes described by Wilson (1945) on serial passage of type 27 streptococci in mice; in these experiments the C polysaccharide of the streptococcus was lost, but type specificity persisted.

Differences in the morphology and in the serological behaviour of degraded organisms have suggested that these constitute a heterogeneous group, but some antigenic relationship between different degraded strains and between degraded and parent strains was also apparent.

We are much indebted to Dr R. E. O. Williams, Director of the Streptococcal Reference Laboratory, Colindale, and to Drs R. W. Vollum and W. H. H. Jebb of the Public Health Laboratory, Oxford, for valuable suggestions and for serological examination of our strains.

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## Effects of Medium Agitation and Wetting Agents on Oxidation of Sulphur by *Thiobacillus thiooxidans*

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**SUMMARY:** *Thiobacillus thiooxidans* oxidizes elemental sulphur more rapidly in a shaken than in static liquid medium. Various wetting agents either had little favourable effect or inhibited growth in static media at 100 p.p.m. In shaken media, some wetting agents were toxic, but 'Tergitol 08' and 'Tween 80' increased sulphur oxidation. 'Carbowaxes' and propylene glycol had relatively little effect on sulphur oxidation.

Oxidation of sulphur and growth of the strictly autotrophic chemosynthetic sulphur bacterium *Thiobacillus thiooxidans* are more rapid in early stages of development than later. This is due in part to inhibitory effects of the acid produced by oxidation of the sulphur (Starkey, 1925), but more to settling of the sulphur in the culture fluid. Most rapid oxidation in stationary media occurs when the sulphur is floating on the surface of the medium. The sulphur settles out as oxidation proceeds.

Since oxygen and carbon dioxide are required for growth and sulphur oxidation, development might depend on the amounts of these gases in the medium. Starkey (1925) noted that oxidation by growing cultures in static medium was greater when the air pressure was increased, but Vogler & Umbreit (1941) found that at  $pO_2$  values from 10 to 30 % and  $pCO_2$  values from 0.01 to 10 % had little effect on oxidation. With resting cells, a  $pO_2$  value below that in air decreased oxidation of elemental sulphur somewhat and resulted in a greater decrease in oxidation of thiosulphate; oxidation was more rapid at increased  $pO_2$  values (Vogler, LePage, & Umbreit, 1942).

The amount of sulphur surface in contact with the medium affects sulphur oxidation. The finer the particle size (Frederick & Starkey, 1948; Starkey, 1925; Vogler & Umbreit, 1941), the greater the concentration of sulphur (Frederick & Starkey, 1948; Waksman & Starkey, 1923), or the greater the amount of surface of liquid medium in contact with a unit of sulphur (Vogler & Umbreit, 1941), the more rapid is the oxidation of sulphur.

This report gives the results of some studies on the effects of agitation and wetting agents on sulphur oxidation by *Thiobacillus thiooxidans*.

### METHODS

A mineral salts medium (Starkey, 1925) containing elemental sulphur (flowers of sulphur) was used in 100 ml. portions in 250 ml. Erlenmeyer flasks. The results recorded are averages of two or more replicates. The incubation

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temperature was 28°. Unless otherwise indicated, shaken flasks were incubated on a rotary shaker that made approximately 200 r.p.m. describing a circle about 5 cm. in diam. The acidity of the culture solutions resulting from oxidation of sulphur to sulphate (titre) is the ml. 0.1N-alkali required to neutralize the acid in 5 ml. culture liquid.

The following wetting agents were tested: 'Tergitol Wetting Agent 08' (Aqueous solution, 38 %); sodium octyl sulphate, an anionic substance from the Carbide and Carbon Chemical Corp. 'Tween 80'; polyoxyethylene sorbitan mono-oleate, a non-ionic substance from the Atlas Powder Co.; 'Alconox' an alkyl naphthalene sulphonate with polyphosphate, an anionic substance from Alconox Inc.; 'Nacconol NRSF' an alkyl benzene sodium sulphonate, an anionic substance from the National Aniline Division; tri-ethylaniline lauryl sulphate, a cationic substance from the Onyx Oil and Chemical Co.; 'Wetsit Conc' an alkylated aromatic sulphonate, an anionic substance from the Jacques Wolf and Co.

## RESULTS

### *Influence of shaking*

The results of one of six experiments on the effects of shaking on oxidation of sulphur are shown in Fig. 1. Sulphur was oxidized more rapidly in shaken media and the effect of shaking was greater the longer the incubation period. Similar results were obtained in all cases, but they varied quantitatively in that the finer the sulphur particles were the more rapid was the oxidation. Rotary shaking resulted in more rapid oxidation than reciprocal shaking, for in the latter case the sulphur accumulated in the necks of the flasks above the surface of the medium. In the stationary flasks practically all of the sulphur was suspended on the surface of the medium initially. This sulphur settled as the culture grew until, after 2 weeks, most of the unoxidized sulphur had settled. Oxidation of submerged sulphur is exceedingly slow in stationary media.

### *Influence of wetting agents*

In most cases the wetting agents caused the sulphur to settle to the bottom of the flasks. This occurred with 'Tween 80', 'Nacconol', triethylaniline lauryl sulphate (TLS), and 'Wetsit'. With 'Alconox' most of the sulphur settled. With 'Tergitol 08' most remained on the surface. The wetting agents had no effect on the pH values at the concentrations used. In static media there was no growth in the presence of 100 p.p.m. of 'Nacconol', 'TLS', or 'Wetsit' and very little growth in the presence of 'Alconox' or 'Tween 80' (Table 1). The culture developed normally in the medium with 'Tergitol 08'.

In shaken media the rate of oxidation of sulphur was increased by 'Tergitol 08' and 'Tween 80', and to some extent by 'Alconox'. The increase sometimes exceeded 100 % with 'Tergitol 08' and 'Tween 80'. 'Tergitol 08' had no effect at 10 p.p.m., but increased the oxidation rate at concentrations of 25–1000 p.p.m. (Fig. 2). An initial inhibitive effect of 1000 p.p.m. disappeared by the 12th day. 'Tween 80' increased the rate of sulphur oxidation at 50 and



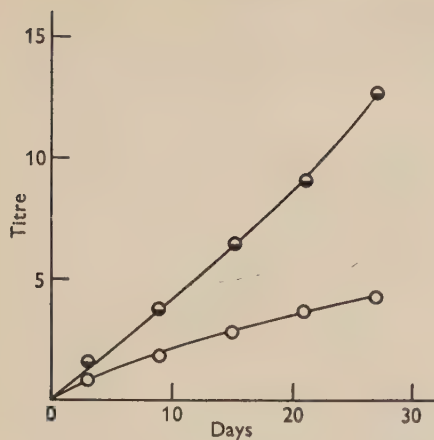


Fig. 1

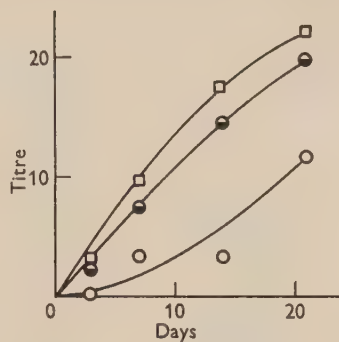


Fig. 3

Fig. 1. Oxidation of sulphur by *Thiobacillus thiooxidans* in shaken (●—●) and static (○—○) media.

Fig. 3. Influence of 'Tween 80' on rate of oxidation of sulphur by *Thiobacillus thiooxidans* in shaken media. ○—○, control; ●—●, 50 p.p.m.; □—□, 250 p.p.m.

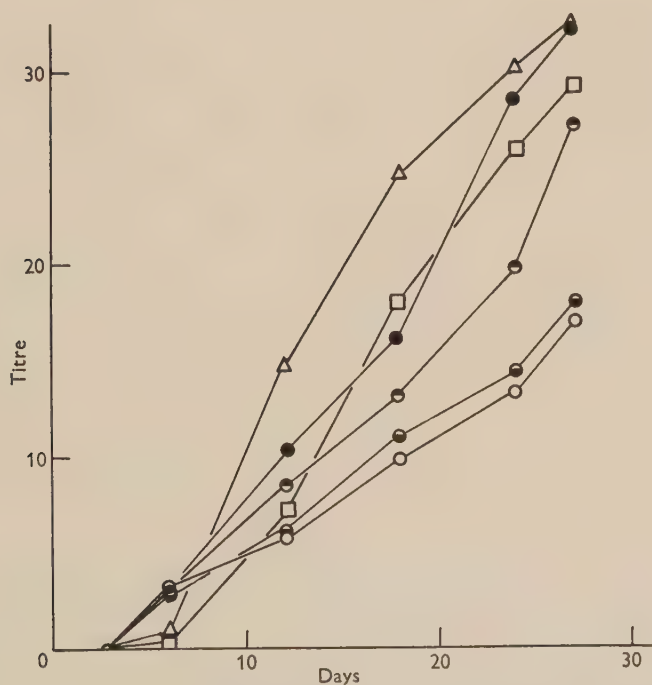


Fig. 2. Influence of concentration of 'Tergitol 08' on rate of oxidation of sulphur by *Thiobacillus thiooxidans* in shaken media. ○—○, control; ●—●, 10 p.p.m.; ●—●, 25 p.p.m.; ●—●, 100 p.p.m.; △—△, 500 p.p.m.; □—□, 1000 p.p.m.

250 p.p.m. (Fig. 3). Some increase was also noted with 'Alconox' at 25 and 100 p.p.m., but growth was inhibited completely at 500 p.p.m. (Table 2). 'Nacconol', 'TLS', and 'Wetsit' prevented growth at 100 p.p.m. in shaken as well as in stationary media.

Table 1. *Influence of wetting agents on sulphur oxidation by Thiobacillus thiooxidans in static media*

Wetting agent	Concn. (p.p.m.)	Titre (see text) after various incubation periods (days)						
		3	7	12	14	15	21	27
Untreated	—	0.8	—	2.4	—	2.8	3.6	4.3
'Alconox'	100	0	—	0	—	0.1	0.4	0.7
Untreated	—	0.3	0.9	—	1.4	—	2.2	—
'Tergitol 08'	100	0.2	1.7	—	2.3	—	2.5	—
'Tergitol 08'	500	0.2	0.6	—	1.2	—	1.3	—
'Tween 80'	50	0.1	0.2	—	0.5	—	0.8	—
'Tween 80'	250	0.1	0.2	—	0.5	—	0.8	—

Table 2. *Influence of concentration of Alconox on rate of oxidation of sulphur by Thiobacillus thiooxidans in shaken media*

Concn. of wetting agent (p.p.m.)	Titre after various incubation periods (days)							
	3	6	9	12	15	18	24	27
Untreated	0.2	1.8	2.4	2.9	3.8	4.7	7.1	9.1
10	0.3	2.2	2.4	3.4	4.2	5.3	7.2	8.9
25	0.3	4.1	4.8	5.0	7.4	9.4	10.6	12.5
100	0.1	0.2	0.7	2.1	3.3	4.6	11.8	15.1
500	0	0	0	0	0	0	0	0

#### *Effects of certain glycols*

Brenner & Owades (1954) reported that 'Carbowaxes' (polyethylene glycols) and propylene glycol dissolved sulphur and produced stable colloidal solutions of sulphur when poured into water. Because these materials might increase the solution of sulphur in the culture medium, the effects of 'Carbowax-200', 'Carbowax-600', and propylene glycol on sulphur oxidation were tested. They were mixed with sulphur, heated at 110–120° for 15 min. to dissolve the sulphur, and then added in appropriate amounts to sterile culture media containing the usual amounts of elemental sulphur. The media were inoculated and incubated on a rotary shaker. When the substances containing dissolved sulphur were added to the culture media, cloudy colloidal suspensions of sulphur were produced, but the medium became clear after the cultures had been incubated for a few days, which indicated that the suspensions were unstable. Though the rate of sulphur oxidation was increased somewhat, it is unlikely that the glycols increased the amount of sulphur in solution. We ascribe such favourable effects as were noted to increases in the amounts of finely divided sulphur in the media.

## DISCUSSION

Elemental sulphur was oxidized more rapidly in shaken than in stationary media. This agrees with results of Newburgh (1954) but disagrees with those of Umbreit (1951).

In practically no case did wetting agents increase oxidation of sulphur in static cultures, but they decreased it in some cases because of an effect other than toxicity. This is contrary to the observation (Vogler & Umbreit, 1941) that sulphur which had settled out in media containing 0.001 % aerosol was often oxidized more rapidly than sulphur on the surface. The smaller oxidation rates in static cultures where toxicity of the wetting agent was not involved was associated with settling of the sulphur to the bottom of the flask. The inhibitory effect can be attributed to deficiency of oxygen or carbon dioxide or both where the sulphur is submerged in the medium. The rate of oxidation in shaken cultures containing the wetting agents 'Tergitol 08' and 'Tween 80' not only exceeded that in static cultures containing no wetting agent, but even that in the untreated shaken medium. Since the surface of a sulphur particle that is wetted by the medium is that from which the bacterium derives sulphur, increase in the amount of wetted surface by wetting agents should and did result in more rapid sulphur oxidation.

There would be less opportunity for continued contact between cells of *Thiobacillus thiooxidans* and solid particles of sulphur in shaken than in static media. Nevertheless, the fact that oxidation was more rapid in shaken media does not exclude the possibility that sulphur oxidation depends on this contact. Through contact, a transportable sulphur substance might be produced enzymically on the cell surface, and the activity of the enzyme could be restored by the active cell. This implies a different means of sulphur uptake from that suggested by Umbreit, Vogel & Vogler (1942), who contended that there was contact between the sulphur and fat contained in the bacterial cells. Knaysi (1943) opposed this theory on cytological grounds. The process seems improbable also because there is little likelihood of actual contact between the fat and sulphur; water films on the sulphur and cell wall would separate the two, and the cell wall itself, indicated by Knaysi (1943) and Umbreit & Anderson (1942), would be an additional barrier.

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## Growth Factors for *Corynebacterium diphtheriae* Strain Dundee

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**SUMMARY:** Investigations were continued on the nature of the growth factor requirements of *Corynebacterium diphtheriae* gravis, strain Dundee. Hypoxanthine, or adenine + guanine, or adenine + xanthine, were found capable of replacing concentrates of the growth factor from liver and yeast.

Nucleoside and nucleotide fractions, prepared from electrophoretically-purified ribonucleic acid, were unable to replace the free purine bases. A purine extract was obtained from an active fish-liver concentrate by copper precipitation. In this extract, a large amount of hypoxanthine and a smaller amount of xanthine were found by chromatography to be the major purine constituents; traces of adenine and guanine were also detected. Chromatographic fractionation and spectrophotometric examination of the fractions confirmed the presence of hypoxanthine and xanthine, whose concentrations were estimated.

The purine extract supported less growth than the original fish-liver concentrates. Growth on the purine extract could be reproduced by a mixture of hypoxanthine and xanthine at an equivalent concentration.

The work of Mueller, Klise, Porter & Graybiel (1933) was the starting-point for the study of the nutrition of *Corynebacterium diphtheriae*. They substituted an acid hydrolysate of casein and tryptophan for commercial peptone and produced a basal medium which with slight modification has been used by subsequent workers in this field. Mueller & SubbaRow (1937) showed that there were at least two substances present in certain components of liver or meat extracts which were growth accessories for these organisms and later in the same year Mueller (1937*a, b*) identified the one as pimelic acid and separated the other fraction into two parts, one of which was identified as nicotinic acid whilst the other was shown to be  $\beta$ -alanine. Mueller & Klotz (1938) found that pantothenic acid could replace  $\beta$ -alanine for their test strain, whilst Evans, Handley & Happold (1939) showed that pantothenic acid was a growth requirement for certain gravis strains.

Pappenheimer (1936) produced potent diphtheria toxin on a simple amino acid medium which was based on that of Mueller and then in conjunction with Johnson (1936) demonstrated the significance of iron in toxin production.

Chattaway, Happold & Sandford (1944) and Chattaway, Dolby, Hall & Happold (1949) reported the presence in liver and yeast concentrates of a factor or factors essential for the growth of certain intermediate and gravis strains of *Corynebacterium diphtheriae* on an otherwise chemically defined medium. Working with acid hydrolysates of brewers' yeast they obtained chromatographic data which suggested that the active material was associated with four ninhydrin-reacting substances. Two of these were resistant to acid

hydrolysis and the remaining two appeared to be peptides; these substances were not then fully characterized. The present work was begun with a view to elucidating further the nature of the active material. It was found that hypoxanthine, and certain mixtures of other purines, were able to replace the growth-promoting ability of a fish liver concentrate of active material and the presence of purines was demonstrated in this concentrate.

#### METHODS

*Organism.* The test organism was a strain of *Corynebacterium diphtheriae* gravis, Dundee subtype, and was the organism used previously by Chattaway *et al.* (1949).

*Inoculum.* The organism was maintained on nutrient agar and before testing was subcultured daily for several days on nutrient broth and finally for 24 hr. on fresh blood agar at 37°. Sufficient isolated colonies were transferred to 5 ml. basal medium to produce a faint turbidity beneath the surface. The organisms were dispersed on a lateral shaker for 5 min. and *c.* 0.05 ml. used per 5 or 10 ml. basal medium.

*Conditions and assay of growth.* Two methods were employed: (i) For routine examinations, growth tests were performed in duplicate 6 ×  $\frac{5}{8}$  in. test-tubes containing 5 ml. medium. After inoculation the tubes were incubated at 37° for 72 hr. and growth was estimated, from the size and thickness of the pellicle formed, by the method of Chattaway *et al.* (1949). The extent of growth is expressed numerically as a percentage of the growth produced on a standard medium containing the active factor. (ii) When strict quantitative comparison of growth was desired, growth experiments were performed in 50 ml. Erlenmeyer flasks containing 10 ml. of medium and covered by 30 ml. beakers. The flasks were incubated for 144 hr. at 37°, the contents transferred to 6 × 1 in. Pyrex boiling tubes and centrifuged. The organisms were washed twice with 0.9 % (w/v) saline, once with distilled water and total nitrogen was determined by the micro-Kjeldahl technique, digestion being performed in the tubes. Results are expressed in  $\mu$ g. bacterial nitrogen/10 ml. medium to the nearest 10  $\mu$ g. Each result is the mean of duplicate determinations.

*Basal medium.* This was essentially the same as that of Chattaway *et al.* (1949), with the addition of pteroylglutamic acid, thiamine and cobaltous chloride; hydroxyproline was absent. Since the exact amino acid requirements of the Dundee organism were not known, equal concentrations of each amino acid were used with the exception of glutamic acid (Mueller, 1940) and tyrosine (solubility considerations). Wherever possible synthetic amino acids were used. The basal medium was made up as required from the following stock solutions: (i) Amino acids (mg./l.): DL-alanine, 800; L-arginine, 400; DL-aspartic acid, 800; L-cystine, 400; DL-glutamic acid, 4000; glycine, 400; L-histidine, 400; DL-leucine, 800; L-isoleucine, 400; L-lysine, 400; DL-methionine, 800; DL-phenylalanine, 800; L-proline, 400; DL-serine, 800; DL-threonine, 800; L-tryptophan, 400; L-tyrosine, 100; DL-valine, 800;  $\text{KH}_2\text{PO}_4$ , 2000; NaCl, 4000. (ii) Growth accessories (mg./l.): pyridoxine, 60; nicotinic acid, 20; calcium

pantothenate, 10; riboflavin, 10; thiamine, 10; *p*-aminobenzoic acid, 5; biotin, 0.1; pteroylglutamic acid, 0.1. (iii) Salts (mg./l.);  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $5 \times 10^4$ ;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 100;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 80;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 30;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 10;  $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ , 173. (iv) 2M-sodium lactate (from A.R. lactic acid), 400 ml./l.;  $\text{CaCl}_2$ , 1.0 g./l.

The final basal medium was prepared by mixing 500 ml. (i), 5 ml. (ii), 5 ml. (iii), diluting, bringing the pH to 7.6 and adjusting the volume to 1 l. After dispensing and autoclaving the medium for 10 min. at 10 lb./sq.in. 0.5 ml. of solution (iv)/10 ml. of medium was added aseptically. Growth of *Corynebacterium diphtheriae* (Dundee) was never observed on this basal medium in the absence of the added growth factors under investigation.

*Source of active factor.* An ether-extracted fish-liver concentrate (Yellow Fin Liver Stick Concentrate, Van Camp Laboratories, U.S.A.) was used as a source of the active factor. Addition of 0.01 ml. of this concentrate to 5 ml. basal medium produced a good growth response and was used as a standard comparison.

*Method of detecting purines on paper chromatograms.* Before the apparatus for the ultraviolet photographic technique of Markham & Smith (1949) was assembled an alternative method was sought. The method detailed below was finally adopted; because of its convenience and high sensitivity its use was continued in conjunction with the ultraviolet method.

The descending paper partition chromatographic technique of Consden, Gordon & Martin (1944) was used. Whatman no. 54 paper ( $18 \times 22$  in.) was selected because of its good wet strength and minimal final background colour. The chromatogram is developed with solvent, and after drying in a current of warm air is sprayed with 0.1% (w/v) copper acetate in ethanol, to which was added ascorbic acid (1 mg./ml.) immediately before spraying. The chromatograms were air-dried, heated at  $120^\circ$  for 5 min. and washed in a rapid stream of tap water for about 30 min. After drying, the paper was sprayed with 0.2% sodium diethyldithiocarbamate in a mixture of ethanol/water/ammonia sp.gr. 0.880, (50:35:15; v/v/v). The position of the purines was marked by dark brown spots on a straw-coloured background. Pyrimidines are not detected by this method; the sensitivity to purines was greater than with the technique of Markham & Smith (1949).

*Ultraviolet absorption data.* These were obtained by means of a 'Unicam' S.P. 500 photoelectric quartz spectrophotometer.

## RESULTS

### *The growth-promoting activity of purines*

As a preliminary to fractionation of the active fish-liver concentrate an examination was undertaken of a variety of substances known to be required as nutrients by other organisms, but not previously tested for our organism. The following compounds were tested singly and in various mixtures at the concentrations indicated ( $\mu\text{g./ml.}$ ): 10.0, adenine; 10.0, guanine; 10.0, xanthine; 10.0, hypoxanthine; 10.0, thymine; 10.0, cytosine; 10.0, uracil; 10.0,



orotic acid; 10.0, guanosine; 100.0, glutamine; 100.0, inositol; 0.1, vitamin B<sub>12</sub> (Cyta-men); and  $5 \times 10^{-4}$  ml. Tween 80 (oleic acid ester).

Hypoxanthine, adenine + guanine and adenine + xanthine permitted growth of the Dundee organism for the first time on a chemically defined medium. All the remaining compounds were entirely without activity. The extent of growth of all possible combinations of adenine, guanine, hypoxanthine and xanthine relative to hypoxanthine is shown in Table 1. Except with hypoxanthine, growth tests on the individual purines at concentrations in the range 1–100  $\mu$ g./ml. showed no activity. The inhibitory effect of adenine, guanine and adenine + guanine on hypoxanthine activity is evident from Table 1. Xanthine appeared to aid in overcoming this inhibition and increased growth on the adenine + guanine mixture.

Table 1. *The relative activity of purines and purine mixtures*

These results are based on growth tests performed at a concentration of 10  $\mu$ g. of each purine/10 ml. basal medium. Incubation: 72 hr. at 37°.

Purine added	Relative activity (%)	Purine added	Relative activity (%)
Hypoxanthine (H)	100	A + X	1
Adenine (A)	0	G + X	0
Guanine (G)	0	H + A + G	6
Xanthine (X)	0	H + A + X	100
H + A	12	H + G + X	100
H + G	6	A + G + X	25
H + X	100	H + A + G + X	100
A + G	6		

Apart from guanosine, no other nucleosides or nucleotides were available for testing. An electrophoretically purified sample of commercial ribonucleic acid (Yeast Nucleic Acid, sodium salt, British Drug Houses Ltd.; 2.5 mg./ml.) was used to prepare nucleotide, nucleoside and free purine fractions by modifications of the methods of Levene & Bass (1931) as follows: (i) *Nucleotides*. 5 ml. sodium nucleate solution was sealed with 0.12 ml. ammonia solution (sp.gr. 0.880) and heated at 120° for 30 min. Ammonia was immediately removed by evaporation under reduced pressure and the volume made to 50 ml. with water. (ii) *Nucleosides*. 5 ml. sodium nucleate solution was sealed with 1 ml. ammonia solution (sp.gr. 0.880) and heated at 120° for 3.5 hr. Subsequent treatment was as above for nucleotides. (iii) *Free purine bases*. 5 ml. sodium nucleate solution was mixed with 2 ml. 5N-hydrochloric acid and left overnight at room temperature. Hydrochloric acid was removed by evaporation under reduced pressure and finally over solid potassium hydroxide *in vacuo*. The final volume was 50 ml.

Activity tests revealed growth only when the fraction containing free purine bases was present; the nucleoside and nucleotide fractions were inactive.



*The active material in fish-liver concentrate*

In view of the previously reported activity of ninhydrin-positive material (Chattaway *et al.* 1949), it was necessary to determine whether purines were the active agents in the fish-liver concentrate and, if so, whether they accounted for all of the activity. A modification of the copper-precipitation technique of Vendrely (1947) was applied to the concentrate before and after acid hydrolysis, so as to distinguish between free and bound purines. The modifications, designed to minimize the accumulation of salt which might interfere in subsequent growth experiments, were as follows: (i) replacement of the 30 % sodium metabisulphite solution by water saturated with sulphur dioxide during the precipitation stage; (ii) decomposition of the copper-purine complex with hydrogen sulphide. No difference was observed in the final purine extracts prepared by either the unmodified or modified method.

Sixty ml. of fish-liver concentrate diluted to 250 ml. with water were heated in a boiling-water bath with 30 % by volume of a saturated solution of  $\text{SO}_2$  in distilled water; 10 % (w/v)  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  were added to the stirred solution in the ratio 5:4 until no further precipitation occurred. The precipitate was removed by centrifugation, washed twice with small volumes of distilled water and the washings added to the supernatant fluid which was reserved.

*Precipitate.* The precipitate was suspended in 20 ml. of water, heated to  $100^\circ$  and hydrogen sulphide passed through the suspension for 15 min. The supernatant fluid was preserved and the precipitate resuspended and again treated with hydrogen sulphide. After washing twice with distilled water, the precipitate was discarded and the combined supernatant fluids and washings were evaporated *in vacuo*. The pH value was adjusted to 7.6 and the solution made to a final volume of 60 ml. This solution was referred to as purine extract P1.

*Supernatant fluid.* The supernatant fluid from the above precipitation was heated at  $100^\circ$  to remove sulphur dioxide, freed from copper by hydrogen sulphide and the copper sulphide discarded after repeated washing. The combined supernatant fluids and washings were freed of hydrogen sulphide, the pH value adjusted to 7.6 and the final volume made to 60 ml. A 30 ml. sample was preserved and designated S1. The remaining 30 ml. was made N with hydrochloric acid and heated at  $100^\circ$  for 8 hr. in sealed tubes. The hydrolysate was repeatedly evaporated under reduced pressure to remove the hydrochloric acid and the precipitation process repeated, yielding an extract P2 and supernatant fluid material S2.

Fig. 1 shows the ultraviolet absorption spectra between 230 and 280  $\text{m}\mu$ . of P1 and P2 diluted 1:200 and 1:20, respectively. The curves indicated that the purine content of the fish-liver concentrate was present in the form of the free purine bases. No purines were detected chromatographically in P2 even in concentrated solutions.

Chromatographic examination of P1 in a variety of solvents revealed the presence of large amounts of hypoxanthine, lesser amounts of xanthine, and traces of adenine and guanine which were only discernible in highly concentrated solutions. Reprecipitation of P1 was necessary to remove traces of

ninhydrin-positive material and a copper-reacting spot not corresponding to any of the four purines previously mentioned. This latter is considered in the next section.

#### *Isolation of hypoxanthine and xanthine from P1*

To obtain further evidence that hypoxanthine and xanthine were the major purine constituents of the fish-liver concentrate, a chromatographic separation was performed as follows. Thirty ml. of P1 solution (once-precipitated) was evaporated to dryness, dissolved in 3 ml. water and distributed across 18 × 22 in. sheets of Whatman no. 54 paper in 10  $\mu$ l. spots. The chromatograms were developed with *n*-butanol/diethylene glycol/water (4:1:1) in an atmosphere of

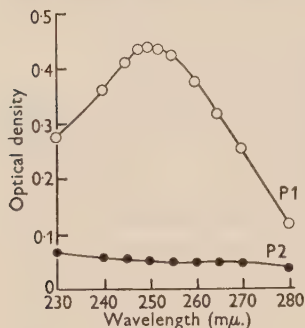


Fig. 1

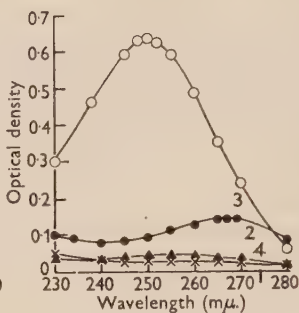


Fig. 2

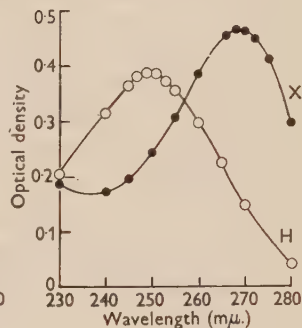


Fig. 3

Fig. 1. The ultraviolet absorption spectra of purine extracts prepared from fish-liver concentrate before (P1), and after (P2), acid hydrolysis. Dilution: P1, 1:200; P2, 1:20. Solvent: water. pH=c. 7.0. Quartz cells, 1 cm. light path.

Fig. 2. The ultraviolet absorption spectra of the copper-positive spots in the purine extract (P1) separated chromatographically. Dilution: 1:100. Solvent: water. pH=c. 7.0. Quartz cells, 1 cm. light path. 1-4=fractions 1-4.

Fig. 3. The ultraviolet absorption spectra of xanthine (X) and hypoxanthine (H). Concentration = 7  $\mu$ g./ml. Solvent: water. pH=c. 7.0. Quartz cells, 1 cm. light path.

ammonia (Vischer & Chargaff, 1948), and marker strips were cut from each edge and developed by the copper method. Four transverse strips were then cut corresponding to four copper-positive spots on the marker strips and each was macerated and eluted by shaking with three successive 100 ml. volumes of dilute ammonia (1 ml. sp.gr. 0.880 ammonia solution to 99 ml. water). The combined eluates from each strip were concentrated to their original volume. Repetition of the chromatography of the four fractions in the same solvent revealed that fraction 1 corresponded to the unidentified, slow-moving material mentioned earlier as being absent from reprecipitated samples of P1; fraction 2 to the xanthine and guanine markers; fractions 3 and 4 to the hypoxanthine and adenine markers respectively. The absorption spectra of the four fractions diluted 1:100 with water is shown in Fig. 2. The well-defined peaks of fractions 2 and 3 at 267 and 250  $m\mu$ ., respectively, closely correspond to the peaks shown by neutral (pH 7.0) aqueous solutions of xanthine and hypoxanthine as determined experimentally (Fig. 3) and to the data of Hotchkiss

(1948). The absence of a well-defined peak in fraction 4 is consistent with the small adenine content of P1, as observed chromatographically. The same argument cannot be applied to fraction 1 since, unlike adenine, a relatively high concentration of the material could be detected on chromatograms and would have been expected to absorb strongly if it were purine. The fact that the material was absent after reprecipitation of the purine extract and could also be detected in large amounts in the fish-liver concentrate, before and after copper precipitation, suggests that its presence was a result of co-precipitation with the purines.

The concentrations of xanthine and hypoxanthine in fractions 2 and 3, respectively, were 220 and 1140  $\mu\text{g./ml.}$  These results are based on standard curves for pure samples of purines measured at the wavelength of maximum absorption and are confirmed by calculation from the absorption data of Hotchkiss (1948).

*Comparison of growth on fish-liver concentrate, purine extract and purines*

A comparison of the growth promoted by the fish-liver concentrate, reprecipitated purine extract P1, hypoxanthine + xanthine and hypoxanthine at comparable levels is presented in Table 2. Growth on the fish-liver concentrate exceeded that on the purine extract, hypoxanthine + xanthine or hypoxanthine

Table 2. *Comparison of growth on fish concentrate, purine extract and purines*

Hypoxanthine + xanthine, and hypoxanthine, were tested at concentrations equivalent to those present in the purine extract and fish-liver concentrate, e.g. 0.01 ml. fish-liver concentrate  $\equiv$  0.01 ml. purine extract  $\equiv$  c. 10  $\mu\text{g.}$  hypoxanthine + c. 2.5  $\mu\text{g.}$  xanthine  $\equiv$  c. 10  $\mu\text{g.}$  hypoxanthine. Incubation: 144 hr. at 37°. Bacterial nitrogen values are average of duplicates to nearest 10  $\mu\text{g.}$

Additions (ml./ml. medium)	Material added to basal medium						Supernatant
	Fish-liver concentrate	Purine extract	Hypo- xanthine + xanthine	Hypo- xanthine	Purine extract + supernatant	Hypo- xanthine + xanthine + supernatant	
	Amount of growth ( $\mu\text{g.}$ bacterial-N/10 ml. medium)						
$1 \times 10^{-5}$	0	0	0	0	—	—	—
$1 \times 10^{-4}$	20	20	20	—	—	—	—
$1 \times 10^{-3}$	70	50	30	30	40	20	0
$5 \times 10^{-3}$	380	170	150	110	—	—	50
$1 \times 10^{-2}$	610	170	170	280	—	—	—

alone. The activity of the mixture of hypoxanthine + xanthine closely paralleled that of the purine extract. The growth observed at the higher level of the supernatant S1 was probably due to traces of purines not precipitated. Upon recombination of the purine extract P1 and the supernatant fluid material S1 it was not possible to reproduce growth on the whole fish-liver concentrate. Thus those growth-stimulating factors responsible for the increased growth on the original fish-liver concentrate above that of the purine extract must have been either lost or destroyed during the precipitation procedure.



Apparently the difference was not due to the presence of inhibitory material added during purine precipitation, since growth on P1 + S1 was approximately the same as growth on P1 alone.

#### DISCUSSION

The addition of purines to a chemically defined medium almost identical with that of Chattaway *et al.* (1949) has thus been shown to allow the growth of *Corynebacterium diphtheriae* strain Dundee. Also, the growth-initiating activity (as distinct from any further growth-stimulatory effect) of a fish-liver concentrate has been correlated with its purine content. These findings differ considerably from the activity of ninhydrin-positive material found by Chattaway *et al.* (1949). One explanation might be that purines and the ninhydrin-positive material are interchangeably active. No evidence in this respect has been obtained in preliminary experiments designed to find a pure protein source of active peptides. An alternative suggestion is that the activity of the material of Chattaway *et al.* was due to the presence of purines. However, comparison of  $R_F$  data reveals no such correlation. Possibly the most plausible explanation is to postulate the presence in the material of Chattaway *et al.* of material involved in the synthesis of purines. Chattaway *et al.* reported different properties of the growth-factor preparations from liver and yeast. Their yeast extract was active at concentrations as low as  $1.7 \times 10^{-4}$   $\mu\text{g./ml.}$ ; the present investigation has shown purine activity only as low as  $0.1$   $\mu\text{g./ml.}$  This difference, by a factor of  $10^4$ , suggests some fundamental difference in function. Whatever the correct explanation may be, it is apparent that the fish-liver concentrate contains some additional material which, whilst having no ability to promote growth in the absence of purines, supplements growth in their presence. Apparently, a requirement for purines has not previously been reported for any other strain of *C. diphtheriae*; an examination of the other gravis strains reported to need the Dundee factor might reveal similar requirements.

It now seems fairly certain that *p*-aminobenzoic acid (*p*-AB) and the folic acid group of factors, in the form of a coenzyme, are concerned in the biological synthesis of purines. It has been suggested (Shive *et al.* 1947) that the coenzyme is responsible for the condensation of a 'one-carbon unit' with 4-amino-5-imidazolecarboxamide, or more probably a derivative of it (Shive, 1950; Woods, 1952), to produce the purine nucleus. Since *p*-AB and pteroyl-glutamic acid were present in the medium throughout the present work, the purine requirement found may have been due to an inability to complete the synthesis of this coenzyme.

Pennington (1942) found that either hypoxanthine or adenine + guanine was required for the growth of *Spirillum serpens* on a basal medium of asparagine + salts. Inhibition of hypoxanthine activity by adenine or guanine was also reported. Thus, whilst it has been pointed out (Brown, 1953) that a wide range of interconvertibility of adenine and guanine by micro-organisms exists, *S. serpens* and *Corynebacterium diphtheriae* strain Dundee appear to be unique in having an absolute requirement for both under certain conditions.

In *Escherichia coli* it seems probable that purine synthesis proceeds by way



of the ribotide of 4-amino-5-imidazolecarboxamide (Greenberg, 1952). In yeast (Williams & Buchanan, 1953) and pigeon liver (Buchanan & Schulman, 1953) this ribotide is converted to inosinic acid. Results with mutant strains of *Aerobacter aerogenes* (Magasanik & Brooke, 1954) suggest that guanine is produced from inosinic acid via a xanthine derivative which is not xanthosine, but is possibly a ribotide of xanthine. In the present study strong growth was observed on hypoxanthine alone, which suggests a facile conversion into nucleic acid adenine and guanine. This might proceed either by direct conversion into adenine or guanine and subsequent incorporation into nucleic acid, or by a preliminary conversion into a hypoxanthine derivative followed by conversion into the corresponding adenine and guanine derivatives. The present results favour the latter alternative because of the lower growth on adenine + guanine, and agrees with the suggestion of other workers that interconversion probably proceeds via the derivatives. Growth on adenine + xanthine was very slow, but does suggest a slight ability for interconversion between xanthine and guanine. In general, the results indicate a similarity in metabolic pathway to that found with *A. aerogenes* (Magasanik & Brooke, 1954). The question of whether the purine derivatives are ribosides or ribotides can only be answered by testing the pure compounds. However, the experiments with hydrolysed ribonucleic acid indicate that ribose derivatives of adenine and guanine are not involved.

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## Taxonomic Rank of Enterobacteriaceae 'Groups'

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**SUMMARY:** The taxonomic value of the different 'groups' into which the Enterobacteriaceae are divided is assessed, and consideration is given to the classification of different groups within the family. No scheme fits the requirements of all workers and two different approaches are made, one for workers in applied bacteriology, and a more logical one for taxonomic purists. For the former scheme it is suggested that common (vulgar) names should be used to describe the groups and their subdivisions. Linnaean binomials are essential for the more systematic scheme, in which the Arizona group is merged with *Salmonella*; Ballerup-Bethesda is combined with *Citrobacter freundii*; Sonne's bacillus and *Bacillus alcalescens* are included in *Escherichia*; Cloaca and Hafnia (32011) are included as species of *Klebsiella*, and Providence (29911) as a species of *Proteus*. Few of these suggestions are new, but the scheme is a classification of a family and not a collection of 'groups'.

In taxonomic studies it is convenient to divide organisms into groups and subgroups, leaving the status of rank for each category to be decided when order appears and the work is nearing completion. Problems of rank are met with in all types of organism; they are attacked in different ways in different disciplines, but even in one branch of microbiology there is no uniformity. Among bacteria certain organisms from the intestines of animals present special problems; their morphological and cultural characters are alike, and resemble those of bacteria found in soil, in water and on plants. Though there is nothing to be said in favour of an ecological grouping of bacteria, in practice ecology may determine the primary grouping: a plant pathologist studies those bacteria found in plants, the water bacteriologist studies essentially similar organisms in water, and the clinical pathologist finds them in human or animal excretions. Often these ecologists work in isolation and develop independent taxonomic schemes.

White (1937), writing on the construction of a taxonomic system for bacteria, contrasted determinative keys with systems based on mutual relations and differences. He said that such a system must develop from multiple foci of intensive study, which would gradually widen until they became confluent; only then could an opinion be formed as to the rational groupings of larger size. Of necessity such a system would be built slowly, but it would be laid on sure foundations. White himself laid many of these foundations and, with Kauffmann, Jordan, St John-Brooks, Schütze and Scott of the original *Salmonella* Subcommittee, paved the way by making an intensive study of one group of organisms. Since then the number of groups studied has increased, and the *Salmonella* Subcommittee of the International Committee on Bacteriological Nomenclature has been replaced by an Enterobacteriaceae Subcommittee (E.S.) which covers many more of the 'multiple foci'. Enlarging



the foci has, as White forecast, resulted in their confluence, and the time seems ripe for an assessment of what are 'rational groupings'.

#### SUBDIVISIONS OF THE ENTEROBACTERIACEAE

Taxonomic ranks are based on the number of shared characters; higher ranks have few characters in common, lower ranks have many, so that the finest subdivisions, or lowest ranks, may be distinguished by only one or two differences. Intensive study of the enteric bacteria has resulted in the use of minute differences to characterize the various subdivisions, but there remains a lack of uniformity of criteria used in characterization of the different ecological groups. Thus the plant pathogens have been studied along the time-honoured lines of morphology and fermentative ability with little study of their serology or of the characters revealed by the newer tests for deaminases and decarboxylases; the water bacteriologists have concentrated their attention on tests to distinguish between so-called 'faecal coli' and those forms believed to have a different source; while the clinical bacteriologists have, until recently, relied largely on antigenic analysis.

The Enterobacteriaceae Subcommittee (Report, 1954*a*) define the family as Gram-negative non-sporing rods, either motile with peritrichate flagella or non-motile, which grow on ordinary media and ferment glucose rapidly, with or without gas production; nitrates are reduced to nitrites. The Subcommittee (Report, 1954*a, b*) recognizes nine groups, *Salmonella*, *Shigella*, *Arizona*, *Escherichia*, *Alcalescens-dispar*, *Klebsiella*, *Bethesda*, *Proteus*, *Providence*, but does not imply that these should be regarded as genera. Kauffmann (1953) would make further subdivisions and add as groups, presumably of equivalent rank, *Cloaca*, *Morganella* and *Rettgerella*. Møller (1954) created another group which he named *Hafnia*. To these should be added *Erwinia* (and perhaps *Pectobacterium*) and the *Prodigiosus* group (reasons for not using the name *Serratia* are given in Cowan, 1956). Even these groups do not include all the enteric bacteria, and the common lactose-fermenting motile forms with IMViC reactions -- ++, which fail to liquefy gelatin, have no place in the scheme.

It seems to me that the 'groups' mentioned are not of equal taxonomic rank, and that it would be profitable to consider what rank should be accorded to each. With this in mind I have drawn up a table which shows the majority reactions of different groups in both the old and newer type of biochemical test (Table 1). Some of the 'groups' shown in Table 1 differ slightly from similar groups of the Enterobacteriaceae Subcommittee Reports; for example *Escherichia freundii* is shown separately and is not included with either the *Escherichia* group or the *Bethesda-Ballerup* group. In recording reactions the *Salmonella* group is shown as gas-producing; this is the reaction of the majority of serotypes, but of course it does not imply that all anaerogenic strains should be classified as *Salmonella typhi*. In the same way *Klebsiella pneumoniae* is shown as VP-positive, although a small proportion of strains is VP-negative.

Before discussing the groups in detail it will be convenient to fix the taxonomic rank of one, to which the others can be referred. *Escherichia*, which is based on *Escherichia coli*, would be the group of choice, but the picture is



Table 1. Reactions of strains of different groups and subdivisions of Enterobacteriaceae

	Providence	<i>Proteus rettgeri</i>	<i>P. morganii</i>	<i>P. mirabilis</i>	<i>P. vulgaris</i>	Hafnia	Cloaca	<i>K. pneumoniae</i>	<i>K. rhinoscleromatis</i>	<i>Klebsiella ozaenae</i>	Bethesda-Ballerup	<i>Escherichia freundii</i>	Other shigellas	<i>Shigella sonnei</i>	A-D group	<i>Escherichia coli</i>	Arizona	Other salmonellas	<i>Salmonella typhi</i>
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gas from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from adonitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from dulcitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from salicin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H <sub>2</sub> S produced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
VP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrates reduced	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glutamic acid decarboxylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on KCN	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on Koser's citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malonate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenylalanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Reactions given are mainly based on Report (1954b), Kauffmann (1954), Edwards & Ewing (1955) and Møller (1954, 1955). Exceptions are *Klebsiella ozaenae*, *K. rhinoscleromatis*, and the decarboxylases of the A-D group, for which detailed descriptions were not found; the results recorded for these organisms are based on tests carried out in the National Collection of Type Cultures (NCTC) by Miss C. Shaw, who also supplied the results of the malonate and phenylalanine tests.

Notation used is that of Kauffmann, Edwards & Ewing (1956), viz. + = positive 1-2 days; ( + ) = delayed positive (3-4 days, occasionally later); × = late and irregularly positive or negative (mutative); - = negative; d = different strains, each consistent in itself, give different reactions (e.g. a single serotype may have strains giving +, ( + ), × or - reactions). Other symbols \* = negative in ferrous chloride gelatin medium, positive when adequate sulphur source available (see Clarke, 1953); † = negative by Hormaeche's method at 45° (personal communication).

complicated by the fact that although the Enterobacteriaceae Subcommittee (Report, 1954*b*), Kauffmann (1954), and Edwards & Ewing (1955) separate *E. freundii* from the Escherichia group, they retain the generic name Escherichia for the species and thus make it appear that the group is an emasculated genus. I shall discuss the Escherichia group presently, and for the moment would prefer to fix the taxonomic rank of the Salmonella group. Most bacteriologists now look upon this group as a genus (see, for example, *Bergey's Manual*, 1948; *Topley and Wilson's Principles*, 1955), and it can, without difficulty, be given generic rank. Therefore, in the sequel, I shall assume that the Salmonella group forms the basis of a genus, *Salmonella*.

#### *Salmonella and Arizona groups*

In the Salmonella group *Salmonella typhi* is in a class of its own; its constant anaerogenic habit and its lack of ornithine decarboxylase distinguish it from the other motile salmonella species or serotypes. Most of the aerogenic salmonellas share a biochemical pattern; this is similar to that of Arizona strains, except that the latter liquefy gelatin, ferment lactose slowly, and do not ferment dulcitol. The malonate test distinguishes Arizona strains from the more typical salmonellas, but some gelatin-liquefying salmonellas (e.g. *S. dar-es-salaam*) are, like Arizona strains, malonate-positive (Shaw, 1956), and thus form a link. Antigenic overlapping is so great that Salmonella diagnostic antisera can be used as substitutes for many of the Arizona antisera (Edwards & Ewing, 1955). As Seligmann & Saphra (1951) and Edwards & Ewing (1955) pointed out, the allocation of some strains to Salmonella or Arizona is quite arbitrary, and the problem is to decide whether the Arizona group is equivalent in rank to Salmonella or only to part of Salmonella.

The Enterobacteriaceae Subcommittee Reports of 1950 and 1953 (Report 1954*a, b*) describe Salmonella and Arizona separately, and this arrangement is followed by Kauffmann (1954) and by Edwards & Ewing (1955). However, this usage has become established because it is more convenient to have two antigenic schemes than to combine them. It seems to me that in a systematic classification the Arizona group is equivalent only to a part of the Salmonella group, of which it should be regarded as a major subdivision, equivalent in rank, *vis à vis* other salmonellas, to *Salmonella typhi*, i.e. to a species. The species thus created, *Salmonella arizonae*, is divisible into the serotypes of the Arizona group.

The nomenclatural problems of *Salmonella* are complex. The name Salmonella received official recognition when the Salmonella Subcommittee was appointed (Report, 1937). The acceptance by the Salmonella Subcommittee (1934) of *S. cholerae-suis* as type species created difficulties that were not foreseen at the time. With increasing knowledge of the permutations and combinations of antigens of the group there is less willingness to regard each combination as a species; these are now viewed as serotypes of a relatively small number of species. Three solutions of the nomenclatural difficulty present themselves: (i) all aerogenic salmonellas can be named *S. cholerae-suis* and individuals distinguished by subspecific epithets or numbers; (ii) the aerogenic

salmonellas can be divided into two species as suggested by Borman, Stuart & Wheeler (1944) and by Kauffmann & Edwards (1952); (iii) each combination of antigens, or serotypes, can be given a common name, as *Salmonella* London. The difficulty arises because of the necessity to report *Salmonella* spp. or serotypes in such a way that their significance will be apparent to a number of people with different professional backgrounds. To a clinician the name *S. cholerae-suis* var. *paratyphi-B* would be extremely confusing, as would *S. cholerae-suis* var. *pullorum* to a veterinarian. Fortunately the needs of the clinician, veterinarian, medical officer of health, sanitary inspector and general practitioner can be met by the use of common rather than scientific names. This is in fact the procedure at present adopted, though the use of italic type and lower-case initial letter for the place name gives the false impression of a specific binomial. For the systematist the suggestions of Borman *et al.* (1944) and of Kauffmann & Edwards (1952) provide the better solution; in considering these proposals it should be borne in mind that the epithet *kauffmannii* has priority over *enterica*.

#### *Escherichia* and *Alkalescens-dispar* (A-D) groups

The *Escherichia* group, as defined in Enterobacteriaceae Subcommittee Reports, consists of organisms classified as *Escherichia coli* and its varieties, including *E. coli-mutabile* and anaerogenic strains. Although the fermentation of lactose is regarded as a main character of the species *E. coli* it is not requisite for inclusion in the *Escherichia* group (Kauffmann, 1954). Should *E. coli* be limited to strains which ferment lactose promptly, another species would have to be designated for the slow fermenters or non-fermenters of lactose that have other characters of the *Escherichia* group; such a species was defined and named *Paracolobactrum coliforme* by Borman *et al.* (1944).

The *Escherichia* group, based on *Escherichia coli* and including both lactose-fermenters and non-fermenters, clearly forms a group of rank equal to the salmonellas. It remains to be decided: (i) whether a species is justified for the late or non-lactose-fermenters; (ii) whether there are species from other groups to be transferred to the genus *Escherichia*.

Kauffmann (1954) would not exclude non-lactose fermenters from *Escherichia coli*, and indeed several strains of his serotypes do not ferment the sugar; the Enterobacteriaceae Subcommittee (Report, 1954*a, b*) also supports the inclusion of the non-lactose fermenting strains. At the present time there is not enough evidence that the lactose fermenters differ significantly from the non-fermenters to justify creating a species based on *Paracolobactrum coliforme* (Borman *et al.* 1944), but, as in all these problems, future work may demand a change in outlook.

A whole 'group' to be included in the genus *Escherichia* is the *Alkalescens-dispar* (A-D) group, composed of anaerogenic, non-motile organisms with O and K antigens of the *Escherichia* group. The recognition of one or two species within this group depends on the importance placed on lactose fermentation; *dispar* is a lactose-fermenter (prompt or delayed) and *alkalescens* does not ferment lactose, but there are many strains difficult to assign to one or other



species; it is, therefore, advisable to combine them, as in the Subcommittee's Reports. *Bacillus alkalescens* and *B. dispar* were both described and named in the same paper (Andrewes, 1918) and priority, decided by the order of their appearance, makes *alkalescens*, changed to *alkalescens* in conformity with the accepted views on transliteration, the correct specific epithet for the combined species. Information on the biochemical reactions of the A-D group is scanty, and I have not found a paper describing their decarboxylases; those observed in a limited number of strains by Miss C. Shaw are shown in Table 1. It is convenient to draw attention here to Sonne's bacillus (*Shigella sonnei*), the indole-negative biochemical counterpart of *Bacillus dispar*; its decarboxylases, except for a quantitative difference in lysine production, are the same as those of *Escherichia coli*, and differ from those of the other *Shigella* species or serotypes. The biochemical and serological characters of Sonne's bacillus are similar to those of the motile coliform C27 (Ferguson & Henderson, 1947; Schmid, Velaudapillai & Niles, 1954), and it seems logical that it should be regarded as an *Escherichia* species, but neither C27 nor Sonne's bacillus has any of the *Escherichia* O antigens 1-130 (Edwards & Ewing, 1955).

Thus, while the transfer of Sonne's organism to the genus *Escherichia* is logical and probably desirable systematically, it is hardly practicable as its membership of the *Shigella* group has become so firmly established. Furthermore, its inclusion in the genus *Escherichia* would be opposed by those who regard the *Shigella* group not as a bacteriological entity but as the dysentery group of bacteria (Bensted, 1956). However, if ever bacterial systematics are allowed to become logical, this is one of the transfers that could profitably be made, and I have made it in the systematic scheme at the end of this paper.

### *Shigella* group

The other *Shigella* spp. form a homogeneous group related to the *Escherichia* group both by biochemical and serological reactions. Their decarboxylase activities are often weak, but the pattern of all shigellas, except Sonne's bacillus, is indistinguishable (Møller, 1954). Many shigella serotypes cross-react with *escherichia* antisera (Ewing, 1953; Edwards & Ewing, 1955) but the majority have, in addition, specific antigens by which they can be recognized, and which justify the genus *Shigella*. It is debatable whether *S. boydii* should be a distinct species or a variety of *S. flexneri*; the serotypes of *S. flexneri* are not biochemically homogeneous and except by serology cannot be distinguished from *S. boydii*; it is probably more systematic to recognize two varieties of *S. flexneri*, namely var. *flexneri* and var. *boydii*.

The removal of Sonne's bacillus from *Shigella* leaves that genus as a group of organisms divided biochemically into two subgroups, but this subdivision based on mannitol fermentation is not without exception; Ewing has found mannitol-fermenting strains of *S. dysenteriae* serotype 3, and non-mannitol-fermenting strains of *S. flexneri* serotypes 1-6, and of *S. boydii* serotypes 3 and 6 (Ewing, 1954; Edwards & Ewing, 1955). In a personal communication, Ewing suggests that any revision of shigella classification should combine all existing species.



The modification of the Enterobacteriaceae Subcommittee's (Report, 1954*a*) classification of shigellas proposed in the fourth edition of *Topley and Wilson's Principles* (1955) is likely to increase confusion; in this three species are recognized in the non-mannitol fermenting subgroup A, and one of them, the Large-Sachs group, is named *S. ambigua*, a name previously attached to Schmitz's bacillus.

Shigella nomenclature has been the subject of an Official Opinion (no. 11) of the Judicial Commission (1954). For the taxonomic scheme suggested here for systematists, there are only two species and Sonne's bacillus is translated to *Escherichia*. However, it is realized that neither clinicians nor clinical pathologists would welcome this improvement in its systematic position, and for their specialized use the name *S. sonnei* would be retained.

#### *Bethesda-Ballerup and freundii group*

The Enterobacteriaceae Subcommittee (Report, 1954*b*) regard the Bethesda group as the slow lactose-fermenting counterpart of the indole-negative cultures classified as *Escherichia freundii*, and include both lactose fermenters and non-fermenters in the 'Bethesda (Bethesda-Ballerup) group'. Kauffmann (1954) used the same arrangement but designated the group as a species, '*Escherichia freundii* (including Bethesda-Ballerup)'. The decarboxylases of the group are unlike those of the *Escherichia* group, and in these, as in the KCN test, the reactions of the group more closely resemble those of the *Klebsiella* group. The lactose fermenters form the two species *Citrobacter freundii* and *C. intermedium* (Werkman & Gillen, 1932), distinguished mainly by H<sub>2</sub>S production; under suitable conditions both are H<sub>2</sub>S-positive (Clarke, 1953) and I have combined them in Table 1. Antigenic overlapping between the *Escherichia* serotypes and *Escherichia freundii* seems to be unusual, but a Bethesda strain related to *Escherichia* serotype O111:B4 has been described (Ørskov, Schmid & Velaudapillai, 1953). Kauffmann (1954), in his table 1, shows *E. freundii* as a species of *Escherichia*, but in his table 3, which is based on the KCN test and decarboxylases, it is grouped with *Klebsiella*, *Cloaca* and *Hafnia*, all of which are KCN positive and can use citrate as a carbon source; in these characters they differ from *E. coli*. There seems to be good reason for including *E. freundii* in the Bethesda group, and for excluding the combined group from *Escherichia*, according it equal (i.e. generic) rank with that group (Kauffmann, 1953). This solution raises a minor problem of nomenclature best disposed of here: *E. freundii* is a synonym of *Citrobacter freundii* (Braak) Werkman & Gillen (1932), and this name antedates *Bethesda ballerupensis* Kauffmann & Edwards (1952) and *Bethesda freundii* (Kauffmann, 1953). Consequently, the generic name valid for the group is *Citrobacter* Werkman & Gillen, and the type species is *Citrobacter freundii*. The Bethesda-Ballerup organisms, which are slow or non-fermenters of lactose, could correctly be given the name *Citrobacter ballerupensis* Kauffmann & Edwards, nov.comb., although such a segregation of lactose fermenters and non-lactose fermenters is not consistent with the policy developed so far in this classification.

*Klebsiella, Cloaca and Hafnia groups*

The *Klebsiella* group is primarily divided by differences in the capsular antigens. Motile forms are excluded (Report, 1954*b*), but neither Kauffmann (1954) nor Edwards & Ewing (1955) regard this as a satisfactory solution of a difficult problem. Edwards & Fife (1955) found that 19 motile strains gave Quellung reactions with *klebsiella* antisera; 17 of these strains liquefied gelatin and all had the general biochemical reactions of the 128 motile strains studied. Edwards (Edwards & Ewing, 1955; Edwards & Fife, 1955) would redefine the genus *Aerobacter* with *Aerobacter cloacae* as the type species; I shall discuss this proposal presently.

If, for the moment, we restrict our consideration to the non-motile forms (i.e. the *Klebsiella* group of the ES reports) the majority of strains are aerogenic and have the IMViC reactions -- ++, but there is a sufficiently large proportion of anaerogenic strains, and strains with different IMViC reactions (Ørskov, 1955) to suggest that the group might be divided on the basis of these reactions. The aerogenic -- ++ forms consist of strains which are typical of *Aerobacter aerogenes* and of Friedländer's bacillus (*Klebsiella pneumoniae*) and, as these cannot be distinguished either by biochemical or serological tests, (Report, 1954*b*) they are now usually considered together, often without species designation (Kauffmann, 1953, 1954; Edwards & Ewing, 1955; Edwards & Fife, 1955). It will be less confusing to say at once that if we accept the *Klebsiella* group as a genus, these aerogenic -- ++ organisms form a species whose correct name is probably *Klebsiella pneumoniae-crouposae* (Zopf) nov.comb. (see Cowan, 1954) but which has become well established as *Klebsiella pneumoniae*, by which name I shall refer to it here.

Strains with other IMViC reactions are distributed among many capsule serotypes but are especially frequent in types 1, 2, 3, 4 and 5; Edwards & Fife (1955) found 81 of 173 strains of these serotypes were MR-positive, VP-negative, and of these 52 of 59 were types 4 and 5. It is not easy to determine from published data the distribution of anaerogenic strains among the different serotypes; for strains of known serotypes examined in this Collection (NCTC) the frequency was as follows:

Serotype	Aerogenic	Anaerogenic
1	9	2
2	15	3
3	7	6
4	17	3
5	8	0
6	9	0

Serotype 3 anaerogenic strains have other characters which segregate them from other *klebsiella* strains (Goslings, 1934), and these undoubtedly represent *Klebsiella rhinoscleromatis*, which can be described as citrate-negative, lactose-negative, malonate-positive and lysine-negative, whereas the aerogenic strains of type 3 are lactose-positive and lysine-positive.

*Klebsiella ozaenae*, represented by types D, E and F of Goslings & Snijders

(1936), or types 4, 5 and 6 in the newer terminology, are either aerogenic or anaerogenic, MR-positive, VP-negative, citrate-positive and malonate-negative.

The motile strains with IMViC reactions — — + + may or may not liquefy gelatin. In describing *Bacillus cloacae*, Jordan (1903) reported that all strains were actively motile, that most liquefied gelatin slowly, that 14 of 21 strains fermented lactose, but often this was slow. Kauffmann (1953, 1954) would resurrect the genus *Cloaca*, Castellani & Chalmers (1919), for these organisms, but Edwards & Fife (1955) prefer to redefine *Aerobacter*, basing it on a new type *A. cloacae*. Another possibility not considered by these authors is to make the motile forms, both liquefying and non-liquefying, a species of *Klebsiella*, i.e. *Klebsiella cloacae* (Jordan) nov.comb., and this is what I propose to do here. This has the advantage that motile and liquefying strains which react with *klebsiella* capsule antisera can be typed as *Klebsiella* species, whereas it would be confusing to describe a strain as *Cloaca cloacae* *Klebsiella* type X, or *Aerobacter cloacae* *Klebsiella* type Y. Unfortunately this kind of designation has sometimes to be made to describe certain antigenic overlaps, e.g. some strains of *Escherichia coli* have salmonella antigens.

Stuart's 32011 type (Stuart, Wheeler, Rustigian & Zimmerman, 1943; Stuart & Rustigian, 1943) forms part of the species *Paracolobactrum aerogenoides* (Borman *et al.* 1944) and falls within the *Hafnia* group of Møller (1954) with the type species *Hafnia alvei* (Bahr) Møller. These bacteria resemble *Klebsiella pneumoniae* in being IMViC — — + +, KCN-positive, malonate-positive and having a strong lysine decarboxylase; they differ in being motile, lactose-negative, urease-negative and having an ornithine and a weak arginine decarboxylase. They also resemble the *Cloaca* group (*K. cloacae*), of which they might be considered the non-lactose fermenting, gelatin-negative counterpart. Eventually the *Cloaca* and *Hafnia* groups may be combined, but for the moment I propose to regard *Hafnia* as a species of *Klebsiella*, with the name *K. alvei* (Bahr) nov.comb. Thus, I have arranged the motile — — + + forms in two species, *K. cloacae* and *K. alvei*, differing mainly on the fermentation of lactose. There are forms which are gelatin-positive and lactose-negative, by Jordan's circumscription these are regarded as *K. cloacae*, as are the gelatin-negative, lactose-positive motile forms. Consideration was given to the possibility of creating another species for the gelatin-negative, lactose-positive form but as the gelatin liquefaction test must have an arbitrary time limit and is greatly influenced by the type of gelatin used and the method of sterilization (Report, 1956*b*), such a new species would be based on much too flimsy grounds and there would be a likelihood that strains would drift from *K. cloacae* to the new species as they gradually lost their gelatin-liquefying power on subculture.

#### *Proteus and Providence groups*

Within recent years the genus *Proteus* has been defined with urease production as the main diagnostic character (Rustigian & Stuart, 1945; Cook, 1948). However, *Proteus* species are not the only members of the Enterobacteriaceae to produce urease; many strains of *Klebsiella* spp., and some strains of *Escherichia* and *Citrobacter* spp. are urease-positive. A more specific distinguishing



character is the ability of *Proteus* spp. to deaminate phenylalanine (Henriksen, 1950). The test, originally described by Henriksen & Closs (1938), is one of the simplest of biochemical tests and, among the Enterobacteriaceae, is positive only in the *Proteus* and *Providencia* groups.

Fulton (1943) proposed that Morgan's No. 1 bacillus should be made the type of a new genus *Morganella*, a suggestion repeated by Kauffmann (1953) together with the removal of Rettger's bacillus from *Proteus* and the creation of a new genus *Rettgerella*. Kauffmann (personal communication) still prefers to separate Morgan's and Rettger's bacilli from *Proteus*, but in his book (Kauffmann, 1954) he does not press this solution. Proom & Woiwod (1951) would not only remove Rettger's bacillus from *Proteus* but Proom (1955) would combine it with the *Providencia* group. On the other hand, several workers (Singer & Bar-Chay, 1954; Buttiaux, Osteux, Fresnoy & Moriametz, 1954; Shaw & Clarke, 1955) have pointed to the relation between *Providencia* and *Proteus* cultures, although only a few *Providencia* cultures are urease-positive. Buttiaux *et al.* (1954) and Shaw & Clarke (1955) made formal proposals that the *Providencia* group, which is the 29911 type of Stuart (Stuart *et al.* 1943; Stuart, Wheeler & McGann, 1946), should be included in the genus *Proteus*. Buttiaux *et al.* (1954) proposed the very appropriate name *P. stuartii*, but Shaw & Clarke (1955) found that a culture of *Bacillus inconstans* belonged to this group and this strain probably represented the oldest described species of what is now the *Providencia* group. The inclusion of the *Providencia* group in the genus *Proteus* subordinates the urease to the phenylalanine test, a test which, among the Enterobacteriaceae, is specific for the five species of *Proteus* as circumscribed here.

The *Prodigiosus* group consists of those peritrichously flagellated Gram-negative rods which produce a pink or red pigment; several species are described but the criteria on which species separation is made are of doubtful value. Dr E. Hormaeche drew my attention to the fact that in biochemical tests many of these organisms resemble the *Cloaca* group. Finally the plant pathogens have not been considered and have been left out of Table 1 as so little is known of their biochemical reactions; aerogenic strains have, in general, the characters of the *Cloaca* group, but the anaerobic strains are more distinctive.

### CONCLUSIONS

This discussion of the classification and nomenclature of the enteric bacteria clearly shows that a logical and systematic classification would not adequately fill the needs of those who work with this group. These specialists are handling cultures sent to them by bacteriologists in certain applied fields, as clinical pathology and water bacteriology, and the reports of the specialists must be readily understood by an even greater variety of people, some of whom have little or no biological background. Thus, there is unfortunately a need for two classifications, one scientific, the other applied.

The taxonomic scheme on which a scientific classification is based is always subject to change as additional knowledge is gained, and this may be reflected



by changes in nomenclature. To the applied scientist such changes are anathema and it is, therefore, better that he should use a scheme that can easily be modified without a consequent change of nomenclature. This can be best done by using common (or vulgar) names for the bacteria being classified; unfortunately many of these common names have some semblance to the Linnaean binomials used in a systematic scheme. The simplest common nomenclature is based on aetiology or ecology, for example, 'the typhoid bacillus' or 'faecal-coli', but, except for pathogens, the plan rapidly breaks down. A compromise has to be made, as in the scheme for coliform bacteria (Report, 1956*a*) in which latinized binomials are used; some, as *Escherichia coli* and *Citrobacter freundii*, are nomenclaturally correct but others, as *Klebsiella aerogenes*, are a compromise between the usage of water bacteriologists and the correct *K. pneumoniae-crooposae*.

Most of this paper has been concerned with the scientific scheme which attempts to systematize the classification of these organisms, and this is summarized in the third column of Table 2. The other scheme must be a

Table 2. *Correlation of classification and nomenclature of: (1) reports of the Enterobacteriaceae Subcommittee; (2) a compromise scheme for applied bacteriology; (3) a scheme for the taxonomic purist*

E.S. Reports	Compromise scheme	Systematist's scheme
Salmonella	<i>Salmonella typhi</i> (typhoid bacillus) <i>S. cholerae-suis</i> (hog cholera bacillus) <i>S. typhi-murium</i> (mouse typhoid bacillus) Salmonella London	<i>Salmonella typhi</i> <i>S. cholerae-suis</i> <i>S. kauffmannii</i> var. <i>typhi-murium</i> <i>S. kauffmannii</i> var. London
Arizona	Arizona + serotype or antigenic formula	<i>S. arizonae</i> + serotype
Escherichia	<i>Escherichia coli</i>	<i>Escherichia coli</i>
Alcalescens-Dispar or A-D group	Alcalescens-dispar (or A-D) + serotype	<i>E. alcalescens</i>
Shigella	<i>Shigella sonnei</i> (Sonne's bacillus) <i>S. dysenteriae</i> { Shiga's bacillus Schmitz's bacillus Large-Sachs' bacilli } <i>S. flexneri</i> (Flexner's bacilli) <i>S. boydii</i> (Boyd's bacilli)	<i>E. sonnei</i> <i>Shigella dysenteriae</i> <i>S. flexneri</i> var. <i>flexneri</i> <i>S. flexneri</i> var. <i>boydii</i>
Bethesda	<i>Bethesda ballerupensis</i> }	<i>Citrobacter freundii</i>
Escherichia freundii	<i>Citrobacter freundii</i> }	
Klebsiella	<i>Klebsiella pneumoniae</i> <i>Klebsiella</i> + serotype  <i>K. aerogenes</i> (water bacteriology)	{ <i>Klebsiella pneumoniae</i> <i>K. rhinoscleromatis</i> <i>K. ozaenae</i>
Cloaca	<i>Cloaca cloacae</i>	<i>K. cloacae</i>
Hafnia	<i>Hafnia alvei</i> or 32011 group	<i>K. alvei</i>
Proteus	<i>Proteus vulgaris</i> <i>P. mirabilis</i> <i>P. morganii</i> (Morgan's bacillus) <i>P. rettgeri</i> (Rettger's bacillus)	<i>Proteus vulgaris</i> <i>P. mirabilis</i> <i>P. morganii</i> <i>P. rettgeri</i>
Providence	Providence + serotype or 29911 group	<i>P. inconstans</i>

compromise and need not be good systematics. Although it would be difficult to improve on the detail of its Reports (1954*a, b*), the Enterobacteriaceae Subcommittee has never published a comprehensive classification. The first column of Table 2 shows the groups of the E.S. Reports, and the second column shows a slight re-arrangement which occupies, I think, a position midway between the working groups of the Subcommittee and the systematist's genera and species. Thus, in the compromise scheme, organisms of the *Salmonella* group would be known and reported as *Salmonella typhi*, *S. paratyphi-B*, or *Salmonella* London, the last preferably with an initial capital for the place name and printed in roman. Until the antigenic scheme for the Arizona group is fully integrated with the *Salmonella* group it will be convenient to retain the two. The only major divergence from the nomenclature used by the E.S. Reports concerns the subgroup named *Escherichia freundii*; these organisms are clearly different from the *E. coli* and it would be wiser to adopt now the nomenclaturally correct, *Citrobacter freundii*. In the *Klebsiella* group, a subgroup or species *Klebsiella aerogenes* is suggested for organisms isolated by water bacteriologists, who would create considerable confusion and dismay among the public if they reported a human pathogen (*K. pneumoniae* or Friedländer's bacillus) in a potable water supply. The *Cloaca* and *Hafnia* groups are not further subdivided, and, in this scheme, *Providencia* is separated from *Proteus*.

Applied bacteriologists are not willing to be treated as chaff and blown hither and thither at every change in taxonomic breeze, and it is our duty to recognize them as the producers of seed which provides us, the taxonomic purists, with our plants for further study.

This paper owes any merits it possesses to a series of discussions with many bacteriologists, to all of whom I want to express my thanks; they include Lt.-Col. H. J. Bensted, Brigadier J. S. K. Boyd, Dr R. E. Buchanan, Dr K. P. Carpenter, Dr P. R. Edwards, Dr W. H. Ewing, Dr E. Hormaeche, Dr F. Kauffmann, Miss Constance Shaw, Dr Joan Taylor, Dr E. Windle Taylor and Dr G. S. Wilson. For the views expressed here I am alone responsible.

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## The Growth of *Mycobacterium tuberculosis* in Semi-solid Agar Media

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**SUMMARY:** Further experience of semi-solid agar media has fully confirmed the earlier opinion (Knox, 1955*a*) of their great value for culture of *Mycobacterium tuberculosis*. The media mainly used were those of Kirchner, Fisher and Dubos, made semi-solid with 0.125 % agar and containing serum or bovine albumin. Large inocula of *M. tuberculosis* H 37 Rv strain in Kirchner semi-solid agar gave easily visible growth in 2-3 days when the simple method of viewing described was used; small inocula gave countable colonies in 10-14 days, or in 7-10 days when a hand-lens was used. Maximum or nearly maximum viable counts were reached in about 2 weeks, and the final number of viable organisms recovered was as high as or higher than in any other media used. The medium also gave rapid growth of other strains of *M. tuberculosis* including drug-resistant variants of H 37 Rv, strains of *M. tuberculosis* var. *bovis*, and of B.C.G. and strains isolated from patients' sputa. Semi-solid media are easy to prepare and safe to handle. Contamination rates are low, evaporation does not occur when rubber stoppers are used, and large numbers of cultures can be inoculated and incubated in a small space. Repeated readings can be made without opening the culture tubes. Viable counts and drug sensitivity tests are easy to read and record. For drug sensitivity tests semi-solid media, besides giving, when serial drug dilutions are used, an initial end-point which is as easy to read as with liquid media, also give information not given by liquid media as to the proportion of 'resistant' organisms present in a given culture. Semi-solid agar media are of great value for any work involving speed in culture, drug sensitivity tests or viable counts of *M. tuberculosis*.

In an earlier paper (Knox, 1955*a*) it was shown that semi-solid agar media were of great value for many different types of work requiring rapid and reliable growth of tubercle bacilli. The purpose of the present paper is to give in greater detail the method used and to describe further experiments which have confirmed and extended the original findings.

### METHODS

**Organisms.** The organisms mainly used were the normal drug-sensitive laboratory strain of *Mycobacterium tuberculosis* var. *hominis* H 37 Rv and a laboratory trained variant (R 1) made resistant to 10 µg. isoniazid/ml. in Dubos liquid medium (Knox, 1955*b*). In a few experiments other strains were used. These included two other laboratory trained resistant variants of the H 37 Rv strain: R 3, resistant to 200 µg. streptomycin/ml. and R 4, resistant to 10 µg. isoniazid/ml. and 200 µg. streptomycin/ml.; a bovine strain of *M. tuberculosis* obtained from Professor Robson; a drug-sensitive strain of B.C.G. and a variant of it resistant to 10 µg. isoniazid/ml.; several strains of *M. tuberculosis* var. *hominis* isolated from patients' sputa. These cultures were maintained by

subculture, usually every 3 months, on Lowenstein Jensen medium. Subcultures were made in Dubos & Davis (1946) liquid medium for inoculation into other media.

*Media.* Liquid media used were: Kirchner medium prepared as described by Mackie & McCartney (1953); Fisher medium (Fisher, 1952); Dubos medium (Dubos & Davis, 1946). The first two media were made in this laboratory, the last was supplied by the Southern Group Laboratory (Park Hospital, Hither Green, London, S.E. 13). Semi-solid media were made by adding to these liquid media agar in a final concentration of 0.125 g./100 ml., except in experiments in which the agar concentration was deliberately varied. Horse serum (supplied by Burroughs Wellcome and Co.) sterilized by Seitz filtration was added aseptically to Kirchner or Fisher medium to give a final concentration of 10%, and bovine serum albumin fraction V (0.35%) to Dubos medium which already contained Tween 80 (0.05%). For use in experiments, the semi-solid agar media were heated to 100° to melt the agar and cooled in a water bath at 48°. Horse serum or bovine serum albumin was added at this temperature immediately before the medium was distributed into sterile  $6 \times \frac{5}{8}$  in. test tubes plugged with cotton wool and kept in racks in a water bath at 48° ready for inoculation. Other media used were Kirchner, Fisher, and Dubos media with 1.5% agar, Hirsch's charcoal medium with 1% agar (Hirsch, 1954) and glycerol blood agar with 50% blood similar to the medium described by Tarshis (1953). These media (except for the liquid Dubos base) were made in the laboratory and distributed for use in Petri dishes. In addition, Lowenstein Jensen slopes in screw-capped bottles were supplied by the Southern Group Laboratory.

*Method of inoculation.* Cultures of *Mycobacterium tuberculosis* in Dubos liquid medium were diluted, usually in tenfold steps, in distilled water unless otherwise stated. Inoculation was by means of dropping pipettes calibrated to deliver 0.02 ml./drop. Each tube containing semi-solid agar was removed from the 48° bath, inoculated and placed in a rack on the bench ready for incubation at 37°. Usually before incubation, or sometimes after overnight incubation, cotton-wool plugs were removed and replaced by rubber bungs or the rubber seals made for viable counts in roll tubes by Astell Laboratory Service Co. Ltd., 172 Brownhill Road, London, S.E. 6. These prevented evaporation as efficiently as rubber bungs and were considerably easier to handle in large numbers. Cultures were incubated at 37° in air.

*Examination of cultures.* Inoculated tubes were examined after 2-3 days and at intervals afterwards. Early growth was made more easily visible by placing each tube to be examined in xylol or ethanol in a beaker or similar container. This had the effect of cleaning the outsides of the culture tubes and eliminated the optical effects of scratch-marks and troublesome reflexions from the walls of the tubes (Knox, 1955*a*). A special viewing-box was made, suitable for routine observation of large numbers of tubes. It had two compartments, an upper in which the xylol-containing beaker was suspended, and a lower containing an electric light bulb which threw its light through the bottom of the beaker obliquely upwards on to the culture tubes to be examined.

The relative position of the beaker and of the light source could be adjusted, so as to give the best oblique illumination for viewing. Cultures were viewed either with a rectangular lens supplied by Britex (Scientific Instruments) Ltd. 329 High Holborn, W.C. 1, giving a magnification of about  $\times 2$ , or with a hand-lens ( $\times 6$ ), or by naked eye. Cultures were usually examined at intervals up to 3 or 4 weeks, but for special purposes longer periods of incubation were used. For microscopic examination, colonies in semi-solid agar were removed with a Pasteur pipette and a drop was placed on a glass slide. A strong straight wire was used to break down the colonies as much as possible and to remove lumps of agar. After drying and fixing by slight heating a Ziehl-Neelsen stain was made in the usual fashion.

## RESULTS

### *Growth of Mycobacterium tuberculosis H 37 Rv in Kirchner's medium with 0.125 % agar*

*The standard medium.* The 'standard' medium for most of the experimental work was Kirchner's medium with 0.125 % agar distributed in 2 ml. amounts in 6 in.  $\times$   $\frac{5}{8}$  in. tubes with rubber stoppers to prevent evaporation. The growth of *Mycobacterium tuberculosis* H 37 Rv at 37° in this medium will therefore be described in some detail. The appearances described are those which were seen with standard conditions of lighting (see Methods).

When a large inoculum was used (0.04 ml. of a 10 to 14-day culture of the H 37 Rv strain in Dubos medium undiluted or diluted 1/10), growth in Kirchner semi-solid agar medium was first visible in 2–3 days. To the naked eye it appeared as a cloudiness of the medium but with a hand-lens ( $\times 4$  or  $\times 6$ ) uncountable numbers of minute colonies could be seen. By the end of 4–5 days, towards the bottom of the tubes the growth still appeared as uncountable numbers of minute colonies now easily visible with the naked eye, but near, and usually 2–3 mm. below, the surface of the agar the individual colonies had coalesced so as to form a band of confluent growth. Growth in this zone continued rapidly. At 10–14 days surface growth began to appear in the form of a pellicle creeping up the sides of the tube, but the minute colonies in the lower part of the medium showed no increase in size. When smaller inocula were used, the appearances were different. With inocula of the order of 100–1000 organisms, minute colonies were first visible at about 5–6 days. On further incubation these increased in size throughout the medium at first, but by 7–10 days the colonies at, or just below, the surface were already becoming larger than the colonies lower down in the agar, and this process continued on further incubation. With inocula of 50 or fewer organisms, as for example with a  $10^{-5}$  or  $10^{-6}$  dilution of a Dubos culture, colonies were first visible to the naked eye at about 7–10 days, though clearly visible with a hand-lens at 4–6 days. On further incubation they continued to increase in size, but not greatly in number, and at 10–14 days they were usually of fairly uniform size throughout the medium. On continued incubation, however, the colonies near the surface grew somewhat larger than those lower down. When, however, the



number of colonies was as small as 10–20 they usually remained fairly uniform in size up to 2–3 weeks, by which time they were  $1-1\frac{1}{2}$  mm. in diameter. Single colonies in Kirchner medium were well defined and roughly spherical in shape, but their surfaces were irregular. Some typical cultures are illustrated in Pl. 1, fig. 1. On prolonged incubation, for 3–4 weeks and longer, secondary colonies began to appear in a track extending vertically downwards from the parent colony (Pl. 1, fig. 2). Ziehl-Neelsen stained films of colonies showed typical acid-ethanol fast bacilli, but no attempt has yet been made to see whether the individual organisms in colonies of different age or at different depths in the agar show any differences in morphology.

Many experiments were performed to investigate the effect of variations in the composition and distribution of the medium, and of changes in the conditions of incubation. These experiments were too numerous to give in full, but the main results will be described.

#### *Variations in composition of the medium*

*Type of agar.* In a few experiments Davis agar supplied by Davis Gelatine (NZ) Ltd., Christchurch, New Zealand, was compared with Oxoid agar (Oxo Ltd., Thames House, Queen Street Place, E.C. 4). In general, growth appeared more rapidly and colonies were larger with Davis agar. In some experiments the number of viable organisms was greater in this agar. Since this gave satisfactory results, the same batch of this was used throughout and no further comparisons were made.

*Concentration of agar.* In liquid Kirchner medium growth of the H 37 Rv strain was first visible in 3–5 days in the form of floccules whose size and rate of growth varied with the size of the inoculum. On the addition of agar, even in as low a concentration as 0.01 %, growth appeared at first in the form of discrete colonies, the number varying with the concentration. These at first remained suspended in the medium but later sank to the bottom of the tube. The gel formed at this concentration of agar was very weak, and in the presence of serum it sometimes contracted into a compact ball containing most of the inoculated organisms. At concentrations of agar between 0.05 and 0.2 % the gel was fairly firm, and colonies remained separate for 2–3 weeks at 37°. At higher agar concentrations the medium was more opaque, growth was much more difficult to recognize and slower in appearing, separate colonies were smaller and viable counts were considerably lower. The optimum agar concentration was 0.1–0.125 %. A typical experiment is illustrated in Table 1.

*The basal medium.* In a number of experiments semi-solid agar medium was prepared with basal media other than Kirchner medium; usually Fisher (Fisher, 1952) and Dubos (Dubos & Davis, 1946) media were tested. The first two media contained 10 % horse serum and the third 0.35 % bovine serum albumin fraction V and 0.05 % Tween 80. Growth of *Mycobacterium tuberculosis* in semi-solid Kirchner or Fisher media was very similar, but quite different in semi-solid Dubos agar. With large inocula, growth in Dubos agar was more diffuse than in the other two media and closely resembled the growth in liquid Dubos medium examined after thorough shaking of the sedimented organisms.



With smaller inocula, colonies appeared in the form of vertical streaks in the Dubos medium and they seldom showed the irregular spherical shape characteristic of their growth in Kirchner semi-solid medium (Pl. 1, fig. 3). Comparisons of the viable counts obtained with these and other media are given in a later section.

Table 1. *Effect of agar concentration on viable-counts of Mycobacterium tuberculosis H 37 Rv*

Medium: Kirchner medium with 10 % horse serum. Readings after 21 days of incubation at 37°.

Agar concentration (%)	Dilution of organisms					
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
	Relative amount of growth (colonies)					
0	++	++	+	+	tr	0
0.01	C	C	SC	unc	14	2
0.05	C	C	SC	unc	18	2
0.125	C	C	SC	unc	25	2
0.2	C	C	SC	unc	15	2
0.4* (rather opaque)	C	C	SC	unc	15	0
0.8* (opaque)	C	C	SC	unc	10	4
					(small)	(small)
1.6* (very opaque)	C	C	SC	few	5	0
					(small)	

C = confluent; SC = semi-confluent; unc = uncountable; \* = growth in these concentrations of agar was mainly at top of medium.

*The effect of serum.* In Kirchner's medium *Mycobacterium tuberculosis* H 37 Rv grew well only in the presence of serum. Growth even of large inocula was erratic without serum or with concentrations of serum up to 0.1 %; at concentrations of serum from 0.5 to 10 % growth was rapid and viable counts were satisfactory. Table 2 shows a typical experiment.

*Distribution of the medium.* Many experiments were performed to find the best type of container and the most satisfactory volume and depth of agar. Too deep a column of agar was unsatisfactory since even with a small inoculum (20–40 organisms) colonies at the bottom remained minute while colonies at the surface grew large. On the other hand, too short a column of agar was also unsatisfactory. With equal inocula, viable counts were higher and colonies were larger and more uniform when the medium was 2–3 cm. deep than when it was only 1 cm. deep or less. From these experiments it was found that the best results were obtained by distributing the medium in volumes of 2 ml., or not more than 4 ml., in 6 in. ×  $\frac{5}{8}$  in. tubes and incubating them with rubber stoppers.

The temperature of the medium at the time of inoculation was important. When the medium was allowed to cool before inoculation the agar, though it never became solid, became too viscous to allow even dispersal of the organisms. When inoculated tubes of semi-solid Kirchner medium were held in the water bath at 48° for 1 hr. after inoculation there was no decrease in the viable

count. A considerable decrease in viable count, however, occurred when tubes were inoculated first and kept at 48° for 1 hr. before the addition of the medium. The most satisfactory results were given when the semi-solid medium after distribution into test tubes was held at 48° before inoculation and each tube immediately after inoculation was removed to room temperature ready for subsequent incubation at 37°. This procedure was therefore adopted as a routine.

Table 2. *Effect of serum concentration on viable counts of Mycobacterium tuberculosis H 37 Rv*

Medium: Kirchner with 0.125 % agar. Inoculum 0.04 ml. Readings after 14 days of incubation at 37°.

Serum concentration (%)	Dilution of organisms					
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>
	Colonies counted or otherwise (see key)					
0	0	0	0	0	0	0
0.01	SC	0	0	0	0	0
0.05	0	0	0	0	0	0
0.1	0	0	0	0	0	0
0.5	C	SC	unc	10	2	0
1.0	C	SC	unc	38	4	0
2.0	C	SC	unc	54	6	0
3.0	C	SC	unc	—	9	1
4.0	C	SC	unc	unc	12	1
5.0	C	SC	unc	unc	9	1
7.5	C	SC	unc	unc	8	1
10.0	C	SC	unc	unc	7	1

C = confluent; SC = semi-confluent; unc = uncountable.

*Conditions of incubation.* In early experiments tubes of semi-solid agar after inoculation were incubated in air at 37° with cotton-wool plugs. It was soon clear that the medium became concentrated by evaporation and increasingly inhibitory to continued growth. With rubber stoppers evaporation was negligible, the medium remained semi-solid indefinitely at 37°, and separate colonies continued to increase in size. At any rate with small inocula, growth was as good in tubes incubated with rubber stoppers for 4 weeks as in tubes which were opened to the air by removing the stoppers once a week and replacing them.

In several experiments parallel sets of inoculated tubes were incubated at 37° with rubber stoppers in an incubator and with cotton-wool plugs in a tin containing air but with only a small outlet to the air of the incubator. No appreciable evaporation occurred in either set of tubes, but in the tubes with the rubber stoppers growth was more rapid and separate colonies were larger than in the tubes in the tin. Further experiments are in progress on the effect of variations in the atmosphere to which the organisms are exposed.

*Viable counts of Mycobacterium tuberculosis H 37 Rv*

The main characters of the growth of *Mycobacterium tuberculosis* H 37 Rv in Kirchner semi-solid agar have already been described. In many experiments designed for different purposes a viable count of the culture was included. For various reasons, results were read after different periods of incubation—in some experiments after 1, 2, 3 and more weeks of incubation, in others at irregular intervals. Each observation was recorded under the appropriate day of incubation together with the final viable count for the same tube. Forty-six experiments are available for analysis. A table was thus constructed showing the average viable counts obtained on different days of incubation and expressed as a proportion of the final viable count. The average number of colonies in 0.04 ml. of a  $10^{-6}$  dilution of a Dubos culture was about 10; the average viable count was therefore about  $250 \times 10^6$  organisms/ml. Up to the ninth day only a small proportion of the colonies finally countable were easily visible to the naked eye, though a much higher proportion could be counted even as early as the seventh day when a hand-lens was used. On the tenth day and after, the number of colonies visible to the naked eye rose sharply and by the end of the second week approached the 100 % level (Table 3). Incubation for 2 weeks is therefore a safe routine for accurate viable counts, though for special purposes or where speed is important, a rough estimate of the number of viable organisms can be given in 7–10 days. Viable counts after more than 4 weeks of incubation were not usually reliable because of the appearance of secondary colonies. Some typical viable counts are shown in Pl. 1.

Table 3. *Viable counts after different periods of incubation expressed as % of the final viable count*

Naked eye counts of colonies recorded.

Incubation period (days)	Proportion of final viable count (%)
Up to 7	2
8–10	14
11–13	80
14	92
15–18	97

*Comparison with other media*

Viable counts of *Mycobacterium tuberculosis* H 37 Rv were performed in a number of different media. Among those tested were the Kirchner, Fisher and Dubos media with 0.125 % agar (in 6 in.  $\times$   $\frac{5}{8}$  in. tubes), the same media with 1.5 % agar in Petri dishes, Tarshis (1953) blood glycerol medium (without penicillin), Hirsch (1954) charcoal medium with 1 % agar in Petri dishes, and Lowenstein Jensen medium as slopes in screw-capped bottles. Some of the results are shown in Table 4. It is clear that semi-solid Kirchner and Fisher media gave counts as high as any of the standard media tested. In addition, growth in semi-solid Kirchner or Fisher media was easily recognizable earlier than in any of the other media, and viable counts were much easier to read.

With the solid media in Petri dishes great difficulty was encountered in avoiding contamination or dehydration, especially when cultures were examined at intervals during incubation. These difficulties could be overcome with elaborate precautions but the tubes of semi-solid agar were much easier to handle in large numbers than Petri dishes, cultures could be repeatedly inspected without exposure to risk of contamination and they gave a very low contamination rate when ordinary bacteriological technique was used.

Table 4. *Viable counts of Mycobacterium tuberculosis H 37 Rv in different media*

Medium	(a) Plates or tubes inoculated (no.)	(b) Plates spoilt by contamina- tion, etc. (no.)	(c) Successful plates (no.)	(d) Colonies counted (no.)	(e) Mean no. of colonies (d/c)
Fisher (1.5 % agar)	43	14	29	508	17
Kirchner (1.5 % agar)	40	8	32	257	8
Hirsch medium (1 % agar)	38	10	28	635	23
Tarshis blood medium (1.5 % agar)	29	2	27	884	33
Fisher (0.125 % agar)	40	1	39	1117	28
Kirchner (0.125 % agar)	37	1	36	1164	32
Dubos (0.125 % agar)	30	0	30	685	23
Lowenstein Jensen	10	0	10	247	25

*The growth of other strains of Mycobacterium tuberculosis in semi-solid agar; drug-resistant variants of H 37 Rv strain*

*Isoniazid-resistant strains.* The strain mostly used was the R 1 strain which was resistant to about 10  $\mu$ g. isoniazid/ml. in Dubos liquid medium, showed dependence on haemin in Fisher's medium and was catalase negative (Knox, 1955*b*). It grew well and rapidly from large inocula in the standard semi-solid Kirchner agar with serum, though when Dubos cultures of equal age were used for inoculation it grew a little more slowly than the isoniazid-sensitive strain and the final viable counts were usually considerably lower. Comparable inocula of viable organisms grew however at about the same rate with both strains. The resistant strain grew more uniformly and for a greater distance below the surface than the sensitive strain when equally large inocula were used. Like the sensitive strain, it grew poorly or not at all in Kirchner semi-solid agar without serum, but it required at least 10 % (v/v) serum for optimal growth. Other isoniazid-resistant strains of *Mycobacterium tuberculosis* var. *hominis* also grew well in semi-solid Kirchner agar with serum. These included the R 4 variant of the H 37 Rv strain resistant to 10  $\mu$ g. isoniazid/ml. and 200  $\mu$ g. streptomycin/ml.; the others were patients' strains which had developed isoniazid resistance during treatment.

*Streptomycin-resistant strains.* These include the R 3 strain resistant to 200  $\mu$ g. streptomycin/ml., the R 4 strain mentioned above, and several streptomycin-resistant strains isolated from the sputum of patients during treatment.



These all gave good and rapid growth in the standard semi-solid Kirchner agar with serum.

*Mycobacterium tuberculosis* var. *bovis* grew well in Kirchner semi-solid agar with serum.

The B.C.G. strain grew more slowly than the H 37 Rv strain but when  $10^{-4}$  dilutions of Dubos cultures were used separate colonies were visible in Kirchner semi-solid agar with serum in 10 days to 2 weeks, and could be accurately counted in 3 weeks or less; this period was considerably shorter than on Lowenstein Jensen medium. Growth was quicker when glycerol was replaced by glucose. The medium was used successfully for viable counts on freeze-dried cultures of B.C.G.

*Sputum concentrates.* Specimens of sputum, from patients with pulmonary tuberculosis, homogenized by the alkali method, were inoculated into semi-solid Kirchner agar with penicillin or malachite green. Excellent growth was obtained even when the number of organisms was small. The results will be more fully reported elsewhere.

#### *Drug sensitivity tests with cultures of Mycobacterium tuberculosis*

Many drug sensitivity tests were carried out in semi-solid agar media both with cultures of *Mycobacterium tuberculosis* and with sputum concentrates inoculated directly.

*Isoniazid sensitivity tests.* These were compared in the liquid media of Kirchner, Fisher and Dubos, and in the same three media with the addition of 0.125% agar. In all these media the initial end-point with the H 37 Rv strain read after 3–4 days of incubation or less was the same, namely, about 0.01–0.05  $\mu$ g. isoniazid/ml., but on continued incubation differences began to appear. Of the liquid media, Fisher medium showed the smallest shift in end-point, Dubos medium showed the steady shift described in an earlier paper (Knox, King & Woodroffe, 1952), while the Kirchner medium showed a rapid shift within 1–2 weeks, to sometimes as high as 10  $\mu$ g./ml. Much of this shift is known to be due to inactivation of isoniazid which occurs in uninoculated media and which is more rapid in Kirchner medium than in either of the others, but liquid media gave no information as to the number of organisms involved in this delayed growth. With semi-solid agar medium it was possible to see the number of colonies developing in increasing drug concentrations. Subcultures from such colonies gave inconsistent results when tested for drug sensitivity. Sometimes they showed considerably increased isoniazid resistance; sometimes despite the fact that the colonies had developed in tubes which originally contained high isoniazid concentrations, the subcultures showed apparently normal sensitivity; sometimes they behaved like mixed cultures, showing for example in Dubos medium apparently normal sensitivity when readings were made after 5–7 days of incubation but showing a much more rapid 'shift' in the end-point on longer incubation.

Isoniazid-resistant variants of the H 37 Rv strain (strains R 3, and R 4) were inhibited by about 10  $\mu$ g. isoniazid/ml. in semi-solid as in liquid media.

The end-point was sharp and could be read in 3–4 days. It was interesting to note the difference between the isoniazid-sensitive and isoniazid-resistant strains of H 37 Rv in Kirchner medium. Both strains eventually grew in 10  $\mu$ g. isoniazid/ml., and in the liquid medium the initial end-point of the sensitive strain was difficult to determine, but some growth occurred up to 10  $\mu$ g. isoniazid/ml.; in Kirchner semi-solid agar however it could be seen at a glance that only a very small proportion of the organisms in the sensitive strain could grow in 10  $\mu$ g. isoniazid/ml. and that these few organisms were the cause of the rapid shift in end-point which occurred in the liquid medium. On the other hand, it was clear that with the resistant strain all or nearly all the organisms grew in 10  $\mu$ g. isoniazid/ml. and without delay.

*Streptomycin sensitivity tests.* These were performed in the same media which showed the same sort of differences as they did with isoniazid sensitivity tests. In all of them, however, end-points were sharper and there was less shift on continued incubation than with isoniazid.

*p-Aminosalicylic acid (PAS) sensitivity tests.* Some preliminary experiments showed that whereas in liquid Dubos medium and liquid Kirchner medium end-points were ill-defined and difficult to read, in semi-solid Dubos agar and particularly in semi-solid Kirchner agar it was easy to see, from the number of colonies which grow, a fairly narrow range of concentrations of PAS within which growth faded from 'growth equal to the control' to a greatly diminished though still large number of colonies only just visible to the naked eye. With such end-points sensitivity tests to PAS can be read like those for isoniazid and streptomycin, in a few days.

In many tests of sensitivity to isoniazid, PAS and streptomycin the effect of inoculum size was investigated. With all three compounds the size of inoculum affected either the initial end-point read after a few days of incubation or the rate of development of resistant colonies, or both. These effects varied with the medium used. The results of these experiments will be more fully reported elsewhere.

*Cultures of sputum concentrates.* Drug-sensitivity tests performed directly on sputum concentrates gave rapid results with sharp end-points. Many experiments performed to determine the best medium for routine use will be reported separately.

## DISCUSSION

Continued experience of semi-solid media agar has fully confirmed the earlier impression (Knox, 1955*a*) of their great value for the cultivation of *Mycobacterium tuberculosis*. A few minor disadvantages will be discussed first. The most obvious criticism is that oxygen may not diffuse freely enough through the depths of the medium to supply the oxygen demands of the growing organisms. At first sight this criticism appears to be supported by the fact that with large inocula growth is much more luxuriant at or near the surface of Kirchner semi-solid agar than in the depths of the medium, where it can be seen that there are numerous minute colonies which develop in the first few days of growth and which then cease to grow any larger, presumably because

all the oxygen is consumed by the organisms nearer the surface. On the other hand, experiments in which the depth of the column of agar was varied by variation in the size of tube and the volume of medium showed that, in 6 in.  $\times$   $\frac{5}{8}$  in. tubes with 2 ml. or perhaps even as much as 4 ml. of medium, colonies of fairly uniform size developed throughout the medium, at any rate during the early days of incubation, provided the standard technique of inoculation described above was followed, and provided the number of colonies was not greater than a maximum of 40–50. It is clear too that, in the first 2 weeks of incubation, enough oxygen diffuses throughout the medium to give viable counts as high as or higher than the counts given by organisms growing on the surface of several standard solid media where presumably oxygen supply at least in the early days of growth is unlimited. Oxygen in too high a concentration is very inhibitory to *M. tuberculosis* (Moore & Stenhouse Williams, 1909, 1911); this may indeed partly be the reason for the inhibitory effect of some solid agar media. In semi-solid agar in air it has often been observed that growth of *M. tuberculosis* is better a few mm. below the surface than immediately below it, that this effect increases with decreasing volume of medium, and that it is only when the column of agar (in 6 in.  $\times$   $\frac{5}{8}$  in. tubes) is more than 2 in. long that there is failure of isolated colonies to develop in the depths of the medium, presumably through lack of oxygen. Further experiments on the effect of oxygen on growth in semi-solid agar will be separately reported. The delayed diffusion of gases which must occur in semi-solid agar is not necessarily altogether disadvantageous for growth of *M. tuberculosis*. One reason why semi-solid agar media sometimes support the growth of small inocula of organisms which fail to grow in the corresponding liquid media may be that essential metabolites, including perhaps CO<sub>2</sub>, do not diffuse away so rapidly as in liquid media. So far as small inocula of *M. tuberculosis* are concerned, it seems that oxygen diffuses into semi-solid agar as fast as it is required, at any rate in the early days of incubation and that oxygen lack does not exert any detectable effect upon growth until about the third week of incubation. Oxygen deficiency however is probably one reason for the poor growth which occurs in the depths of media with agar concentrations much above 0.4%.

The number of colonies which can be conveniently counted in semi-solid agar medium is not more than 40–50. When the inoculum is diluted in tenfold steps it is sometimes found that a 10<sup>-5</sup> dilution contains too many and a 10<sup>-6</sup> dilution too few colonies. This difficulty is easily avoided, either by using closer dilutions, increasing the number of drops inoculated per tube, or inoculating several duplicates, the number depending on the accuracy required. Sampling of colonies from semi-solid agar for storing or for subcultures is not quite so easy as sampling from the surface of solid media or from liquid media but presents no serious difficulties, except for the preparation of thick suspensions, for which the medium is quite unsuitable.

On the other hand, semi-solid media have certain outstanding advantages. It has been shown that Kirchner semi-solid agar gives very rapid growth of large inocula—growth which is frequently easily recognizable much earlier than in the corresponding liquid medium; it supports the growth of small



inocula and colonies are visible and countable more easily and earlier than in any other media here used.

Because ordinary 6 in.  $\times$   $\frac{5}{8}$  in. test tubes are used it is possible to handle large numbers of cultures at a time, with the use of relatively small volumes of medium and of incubator space. Cultures can be repeatedly examined without in any way exposing them to risk of contamination, contamination rates are very low, and the technique of inoculation is probably much safer to the operator and less liable to be spoiled by contamination than techniques which involve dropping cultures on to the surface of Petri plates exposed to the air.

Because with large inocula growth is easily visible in 2-3 days, the medium is excellent for rapid drug-sensitivity tests. The development of 'resistant' colonies can be followed at intervals during incubation. It is true that these presumptively resistant colonies often showed on subculture lower degrees of resistance than might have been expected from the high concentration of isoniazid initially present in the tubes in which they grew. But the fact that such colonies grow at all in such tubes indicates that even if they are really mixed cultures they do at any rate contain an increased proportion of organisms with resistance greater than normal. With semi-solid agar it is at least possible to obtain a rough estimate of the frequency with which such 'resistant' organisms occur in a given culture as compared with a normal fully-sensitive strain in the same medium. Thus cultures in semi-solid agar media besides having the advantages associated with the usual serial dilution methods in tubes of liquid media give additional information as to the distribution of resistant organisms in the bacterial population. There are, however, considerable differences between the three media we have used. Fisher's medium perhaps tends to underestimate the proportion of resistant organisms in a predominantly sensitive culture, since growth of resistant strains in Fisher medium is erratic even in the presence of serum, unless haemin is added; Kirchner medium, on the other hand, probably gives an exaggerated picture since the growth of large numbers of colonies in this medium may reflect not so much a general increase of resistant organisms as a more rapid destruction of isoniazid in this medium. Dubos semi-solid agar seems to be the most satisfactory for isoniazid-sensitivity tests, since it is possible to give both an early report on the average sensitivity of a culture and a late report on the proportion of (presumptive) isoniazid resistant organisms.

The Dubos semi-solid agar medium has proved useful for the standardization of cultures of *Mycobacterium tuberculosis*. It was through its use at the National Collection of Type Cultures for viable counts on freeze-dried cultures of *M. tuberculosis* that our attention was first drawn to the medium. We have used it successfully for assessing the viability of freeze-dried culture of *M. tuberculosis* H 37 Rr and B.C.G. Its possible value for the standardization of B.C.G. vaccine is now being investigated.

Our thanks are due to Mr C. E. Engel, Photographer to the Medical School, Guy's Hospital, for his valuable advice and help in the photography, and to the Medical Research Council for a grant for technical assistance.





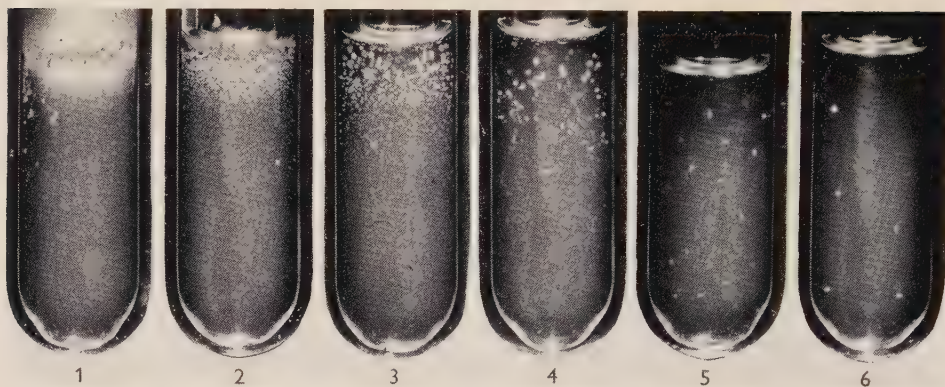


Fig. 1



Fig. 2

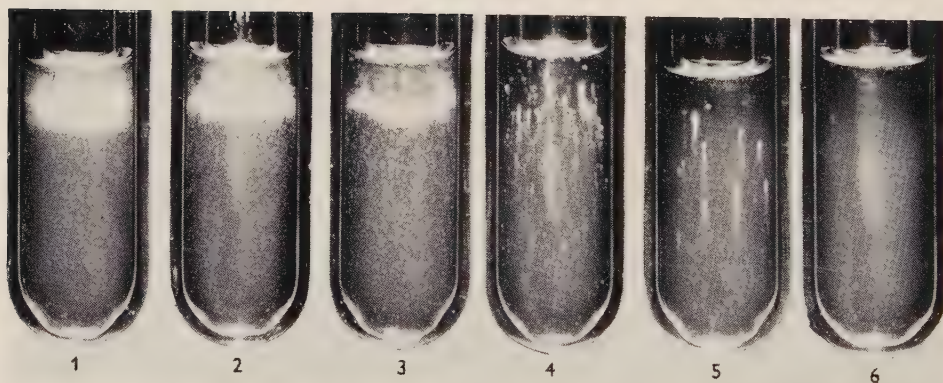


Fig. 3

R. KNOX, E. SWAIT & R. WOODROFFE—*M. TUBERCULOSIS*: GROWTH ON SEMI-SOLID MEDIA

(Facing p. 371)

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## EXPLANATION OF PLATE

- Fig. 1. Viable count of *M. tuberculosis* H 37 Rv in Kirchner semi-solid agar (4 ml. per tube). Incubated 20 days at 37°. Inoculum 0.04 ml. of a 12-day Dubos culture. 1 in 10 dilution in tube 1 and then decimal dilutions to 10<sup>-6</sup>.
- Fig. 2. Culture of *M. tuberculosis* H 37 Rv showing secondary colonies after 28 days incubation at 37°.
- Fig. 3. Viable count of *M. tuberculosis* H 37 Rv in Dubos semi-solid agar (4 ml. per tube). Incubated at 37°. Inoculum as in Fig. 1.

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## Antibiotic Activity of Actinomycetes in Soil as Demonstrated by Direct Observation Techniques

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**SUMMARY:** Direct observation techniques were used to determine the antibiotic effects of eight actinomycete species on *Helminthosporium sativum* in soil and *in vitro*. In actinomycete-inoculated sterilized soils, the inhibition of germination of fungal spores corresponded with the degree of inhibition of the fungus produced by the same actinomycetes in Petri plate culture. The effects of the actinomycetes on the vegetative growth of *H. sativum* in soybean-supplemented soils varied with the individual actinomycetes and, with one exception, were identical with the effects caused by the actinomycete antibiotics *in vitro*. These hyphal changes in soil included: suspension of further mycelial development; lysis; characteristic morphological effects such as stunting, distortion, excessive branching and the formation of hyphal protuberances. Lysis of the fungal hyphae only occurred in the presence of soil and was shown to be due to the combined effect of the antibiotic and some unidentified soil factor. Evidence was also obtained which demonstrated the ability of the actinomycetes to produce antibiotics in unsupplemented soils. Additional proof of antibiotic activity in soil was obtained by using the antibiotic actinomycin and strains of *Streptomyces antibioticus*, the organism responsible for its production. In Petri plate culture, both crystalline actinomycin and the actinomycetes produced a characteristic swelling, distortion and stunting of the vegetative growth of *Helminthosporium sativum*. When introduced into sterile soils, identical morphological changes were evidenced by the fungus in the presence of either the pure antibiotic or the actinomycetes.

Although the ecological significance of antibiotics in soil has often been questioned because of the failure to demonstrate the presence of these substances, the possibility remains that antibiotics are present in amounts too small to be detected, but in sufficient concentration to exert localized inhibitory effects. In the past, the production and accumulation of antibiotics in soil has generally been assessed on the basis of extraction techniques (Gottlieb & Siminoff, 1952; Grossbard, 1952; Gregory, Allen, Riker, Peterson, 1952) and the frequent failure to detect antibiotics may well be due to the inadequacy of these methods (Stevenson, 1956).

Mitchell, Hooton & Clark (1941) were among the first to suggest that direct observation of the growth antagonistic processes occurring in soil would aid in the understanding of the mechanisms of biological control. By using a Rossi-Cholodny slide technique these workers showed the decomposition of *Phymatotrimum omnivorum* by successive groups of soil micro-organisms. Blair (1943, 1945) described the use of similar techniques in following the growth behaviour of *Rhizoctonia solani* under various soil conditions. In the study of fungistasis in the Wareham Heath soils, Jefferys & Hemming (1953) placed



slabs of agar in intimate contact with soil and examined them for antibiotic effects by removing the slabs and seeding with a test organism. In this way, these workers were able to detect localized areas of inhibition. Legge (1952), and Dobbs & Hinson (1953) developed techniques in which test fungi were enmeshed in glass fibre tape or between layers of cellulose film before placing in soil. After a suitable exposure period the tapes or films were removed and examined for antibiotic effects. Chinn (1953) devised a practical buried-slide technique for studying the effects of soil conditions on the germination of fungal spores.

Reilly, Schatz & Waksman (1945) have shown that antibiotic substances may vary greatly in their antifungal effects. An antibiotic may be fungistatic or fungicidal, depending on its concentration. The presence of an antibiotic may also bring about some physiological change or result in the complete lysis and destruction of the fungus. Another phase of antibiosis, namely, the induction of morphological change in the antagonized organism, has recently received considerable attention. Numerous workers (Christensen & Davies, 1940; Lachance, 1951; Tveit, 1953; Rombouts, 1953) have demonstrated the effects of antibiotic substances on germination, hyphal development and morphology of fungi. Brian, Curtis & Hemming (1946) showed that antibiotics can affect the morphological development of fungi in a characteristic and reproducible manner. These workers demonstrated two distinct effects of the antibiotic griseofulvin on the spores and hyphae of *Botrytis allii*: (i) inhibition of growth only at a stage subsequent to germination of the conidia; (ii) a remarkable increase in branching and distortion of the hyphae.

In this present paper, a direct observation technique has been employed in which the specific effects of a number of actinomycete antibiotics in *Helminthosporium sativum* have been used in an attempt to establish the presence and activity of these substances in soils.

#### METHODS

*The actinomycetes.* Eight of the ten *Streptomyces* spp. used in previous studies (Stevenson, 1956) were employed in the present investigation. Two of the organisms, E4 and E63, were identified as strains of *S. antibioticus* and produced the antibiotic actinomycin (Stevenson, 1956). The actinomycetes AA30, AA53, AA24, AA27, XX19 and Y27 remain unidentified.

*Determination of the in vitro effects of the actinomycete antibiotics on spores of Helminthosporium sativum.* The actinomycetes were streak inoculated on plates of Conn's asparagine agar and incubated at 26° for five days. The plates were then flooded up to the actinomycete growth with 2 ml. of potato glucose agar (PG agar) on which was spread 0.5 ml. of a heavy suspension of *H. sativum* spores. The plates were re-incubated and examined under the microscope after 1, 3- and 7-day intervals. Microscopic examination: by means of a sharp scalpel, a small rectangle of agar was removed at right angles to the actinomycete-streak and placed on a clean microscope slide (Pl. 1, fig. 1). The surface of the agar block was flooded with a dilute solution of aniline blue

(in lactophenol) for 2–3 min. and then washed off. Development of the fungal spores was determined by examination of the agar block under a microscope.

*Determination of the in vitro effects of the actinomycete antibiotics on pre-developed hyphae of Helminthosporium sativum.* In order to determine the antibiotic effects on *H. sativum* hyphae, a spore suspension was added to plates of PG agar and incubated for 24 hr. to produce vegetative growth of c. 200–500  $\mu$ . long. Actinomycete plates were prepared in the manner described above. Square agar blocks were removed from the actinomycete plates (from areas known to contain the actinomycete antibiotic) and placed in the centre of plates containing 24 hr. growth of *H. sativum*. These plates were re-incubated and examined at 1-, 3- and 7-days by the methods described for spores (Pl. 1, fig. 2).

#### *Buried-slide techniques*

*Preparation of spore-slides.* A modification of the buried-slide technique of Chinn (1953) was used. Spores of *Helminthosporium sativum* were suspended in Tween 20 and the suspension was filtered through 200-mesh phosphor bronze wire cloth to remove mycelial fragments. The spore concentration of the suspension was calculated from readings with a Turek haemocytometer, and a final concentration of 2,000,000 spores/ml. was obtained by the addition of distilled water. Ten ml. of this suspension was added to 100 ml. sterile 1.5% agar which had previously been melted, cooled and held at 45°.

Ordinary microscope slides, thoroughly cleaned and sterilized, were dipped momentarily into the agar spore suspension, then removed and held horizontally until set. The slides were then inserted vertically about one-quarter of an inch into a shallow layer of sterile soil in the bottom of 3 in. diameter clay pots. After the insertion of the slides, soil alone or actinomycete + soil inoculum was carefully added to the pots. At the end of each specified incubation period (1, 3 and 7 days), the slides were carefully removed from the pots by the use of a flamed spatula. After removal, the slides were rinsed in water to remove adhering soil particles, and the agar film from one side of the slide was wiped off. The slides were then stained (aniline blue-lactophenol) for 2–3 min. and immediately examined under the microscope. The degree of spore germination (%) was calculated on the basis of examination of 25 spores on each of two slides.

*Preparation of slides with pre-developed hyphae.* Spore slides were prepared as described above, but before placing in the soil the slides were incubated for 24 hr. in a moist chamber at 26°. Incubation under these conditions prevented the thin agar film from drying out and allowed the germ-tubes to develop to about 200  $\mu$ . long. The slides were then placed in soil and examined in the usual manner.

*Soils and the preparation of actinomycete inocula.* The soils and the preparation of actinomycete-soil inocula were described previously (Stevenson, 1956).

## RESULTS

*The in vitro effects of actinomycete antibiotics on germination and development of Helminthosporium sativum*

Preliminary experiments were undertaken to determine the antibiotic effects of the eight actinomycetes on spore germination and hyphal development of *Helminthosporium sativum*. By examining spores and hyphae within the normal inhibition zones of the actinomycetes, it was possible to arrange the antagonists into four groups according to the observed effects on the fungal spores and hyphae. Table 1 summarizes the observations made over a period of 7 days at a point midway through the inhibition zone.

Table 1. *Antibiotic effects on spores and pre-developed hyphae of Helminthosporium sativum within the normal inhibition zones of the actinomycetes*

Actinomycete strain	Width of zones of inhibition (mm.)	Effects of antibiotics on	
		Spores	Pre-developed hyphae
AA 30	4 }	80 % germination. Slight inhibition of further development	Hyphae remains normal in appearance. Slight inhibition of further growth
AA 53	10 }		
AA 27	20 }	10–20 % germination. Spores germinate only to the P.E.I. stage (Pl. 1, fig. 3). No further development on continued incubation	Further development suspended. Distortion frequently noted (Pl. 1, fig. 4)
AA 24	22 }		
E 4	15 }	30–40 % germination. Majority of spores germinate to the P.E.I. stage with no further development	Hyphae swollen, distorted and frequently more branched (Pl. 1, fig. 5). Slight increase in growth
E 63	18 }		
XX 19	24 }	Germination completely inhibited	Further development inhibited. Some formation of hyphal protuberances at periphery of zone (Pl. 2, fig. 9)
Y 27	24 }		

\* Zone widths; see Stevenson (1956).

Of the four groups, that made up of the actinomycetes AA 30 and AA 53 exerted the least effect on the fungus. In the presence of the antibiotics of these organisms, only slight inhibition of germination and hyphal development was noted. A considerable diminution in degree of germination occurred with the actinomycetes AA 24 and AA 27. Although 10–20 % germination was noted, the development of the germ-tubes rarely progressed further than illustrated in Pl. 1, fig. 3. This inhibition of development just after germination is referred to as post-emergence inhibition (P.E.I.). In the majority of instances further hyphal development was inhibited in the presence of these two actinomycetes. Some distortion of the more extensively developed mycelia was also noted (Pl. 1, fig. 4). The antibiotic effects of the actinomycetes E 4 and E 63 are distinguishable by inhibition at a post-emergence stage and by the characteristic distortion of the pre-developed hyphae. The typical stunted, swollen and



branched appearance of *Helminthosporium sativum* is illustrated in Pl. 1, fig. 5. The antibiotic effects of actinomycetes XX 19 and Y 27 are characterized by the complete inhibition of germination and the formation of distinct hyphal protuberances on pre-developed hyphae within the antibiotic zone (Pl. 1, fig. 9).

*The antibiotic effects of actinomycetes on spores and pre-developed hyphae of Helminthosporium sativum in acid, neutral and alkaline soils*

A study of the antagonistic effects of the eight actinomycetes on spores and pre-developed hyphae in acid, neutral and alkaline soils was undertaken in view of earlier studies which demonstrated the controlling effects of these organisms on root-rot of wheat (*Helminthosporium sativum*) in these soils (Stevenson, 1956). The actinomycete + soil inoculum was again prepared with 2% soybean meal and the growth habits of the fungus were determined in sterile and non-sterile soils, with and without the addition of this material. The results of these investigations are summarized in Table 2.

The presence of soybean meal in the acid soil caused an adverse affect on the fungus. Although a high percentage germination was recorded for the sterile soil alone, the percentage germination in the sterile soil + soybean mixture was markedly less. No diminution in degree of germination was noted in the neutral and alkaline soils with soybean. This would suggest that the diminished degree of germination in the acid soil resulted from the combined effect of sterilization of the acid soil with the soybean meal. Further investigations demonstrated that total germination of *Helminthosporium sativum* spores resulted when the acid soil and soybean meal were sterilized separately before mixing. Inasmuch as the actinomycete + soil inocula used in these studies were prepared in sterilized soil + soybean mixtures, it is quite possible that the decreased germination and growth of *H. sativum* in the actinomycete-inoculated acid soil was due to the toxic effects resulting from the combined sterilization rather than to antibiotic action. A study was made to determine the antibiotic activity of the actinomycetes in the acid soil when the inocula were prepared with a soil + soybean mixture in which the components were sterilized separately. In all cases 90–100% germination of the fungal spores resulted and there was no inhibition of further growth of the pre-developed hyphae. Since no antagonistic effects of the actinomycetes were noted, it would appear that these organisms cannot adapt themselves to the acid environment of this soil. Results of previous studies also suggested this (Stevenson, 1956).

A low percentage spore germination and the rapid destruction of the developed hyphae are characteristic effects on *Helminthosporium sativum* in non-sterile soils (Chinn, 1953). The addition of soybean meal to the non-sterile soil brings about a higher percentage germination and stimulates more extensive development of the hyphae prior to attack by the natural soil antagonists. Lysis and various forms of hyphal distortion were noted by 24 hr. and generally, after 3 days, only remnants of the vegetative growth remained.

For the most part, the antagonistic effects of the actinomycetes on germination and development of *Helminthosporium sativum* in the neutral and alkaline



soils were identical with those observed on the fungus in the presence of the actinomycete antibiotics *in vitro*. In all cases there was a definite decrease in the development of the fungus in the presence of the actinomycetes, and the

Table 2. *The effects of the actinomycetes on spores and pre-developed hyphae of Helminthosporium sativum after 3 days of exposure in acid, neutral and alkaline soils*

Soil treatments	Acid soil		Neutral and alkaline soils	
	Spores (% germination and degree of growth)*	Pre-developed hyphae	Spores (% germination and degree of growth)*	Pre-developed hyphae
Sterile soil	90–100 % germination. + + + to + + + +	Healthy, extensive growth	100 % germination. + + + to + + + +	Healthy, extensive growth
Sterile soil + 2 % soybean meal	40–50 % germination. + to + +	Healthy, extensive growth	100 % germination. + + + to + + + +	Healthy, extensive growth
Non-sterile soil	30 % germination. + to + +. Germ tubes lysed	No further growth. Majority of hyphae lysed. Distortion evident	10–30 % germination. + to + +. Germ tubes lysed	No further growth. Majority of hyphae lysed. Distortion evident
Non-sterile soil + 2 % soybean meal	98 % germination. + + + to + + + +. Distortion and lysis evident	No further growth. Distortion and lysis evident	85 to 100 % germination. + + + +. Distortion and lysis evident	Majority of hyphae completely lysed. Distortion evident
Actinomycetes AA 30, AA 53†	.	.	80–90 % germination. + + + to + + + +	Healthy, extensive growth
Actinomycetes AA 24, AA 27†	25–40 % germination. + to + +	No further development	35–40 % germination. +. Spores germinated to P.E.I. stage only	No further growth. Majority of hyphae completely lysed (Pl. 1, fig. 6). Some distortion noted
Actinomycetes E 4, E 63†	.	.	50 % germination. + to + +. P.E.I. (Pl. 2, fig. 7) Distortion of more extensive mycelia	No further growth. Hyphae stunted, swollen, with some lysis at hyphal tips (Pl. 2, fig. 8)
Actinomycetes XX 19, Y 27†	.	.	40–50 % germination. +. No further development	Slight increase in growth. Formation of hyphal protuberances (Pl. 2, fig. 9)

\* Growth estimated as follows: + = spore wall ruptured and germ-tube emerging; + + = development of hyphae to about 200  $\mu$ ; + + + = development of hyphae to about 1000  $\mu$ ; + + + + = very extensive mycelial development.

† Sterile soil + 2 % soybean meal, inoculated with the actinomycete indicated.

degree of antagonism displayed by these organisms agreed with the degree of inhibition of the fungus in Petri plate culture. Some of the typical effects of the actinomycetes on *H. sativum* in soil are illustrated in Pl. 1, fig. 6, and Pl. 2, figs. 7, 8.

Although no lysis of *Helminthosporium sativum* was evident in the presence of the antibiotics in the *in vitro* studies, this effect was noted with some of the

actinomycetes in soil. The actinomycetes E4 and E63 frequently lysed the terminal portions of the developing hyphae (Pl. 2, fig. 8), but the complete lysis and destruction of the fungus was peculiar to actinomycetes AA24 and AA27 (Pl. 1, fig. 6). In the case of these latter organisms the lytic effect was generally evident by 24 hr., lysis of the fungus being completed in as little as 3 days. Further experiments established that although the antibiotics of actinomycetes AA24 and AA27 were incapable of exerting a lytic effect on the fungus by themselves, in combination with soil or soil extracts lysis did occur.

The foregoing experiments were repeated in soils to which no soybean meal had been added in the preparation of the actinomycete + soil inocula. In a previous report (Stevenson, 1956) it was found that antibiotics could not be detected by extraction techniques in inoculated unsupplemented soils. By means of the buried-slide technique it was shown that the effects of the actinomycetes on the fungus in these unsupplemented soils were, for the most part, identical with those outlined in Table 2. Some decrease in the degree of antibiosis was indicated by a higher percentage of unaffected spores and more extensive hyphal development.

*The effect of actinomycin on spores and pre-developed hyphae  
of Helminthosporium sativum*

In order further to substantiate the belief that the antagonistic effects noted in soil were due to antibiotics, use was made of the knowledge that the actinomycetes E4 and E63 were strains of *Streptomyces antibioticus* which produce the antibiotic actinomycin. The morphological changes induced by these actinomycetes in soil are very distinctive, and if the antibiotics produced by these organisms are the responsible agents, one would expect similar changes in *Helminthosporium sativum* in the presence of pure actinomycin.

The *in vitro* effects of actinomycin were determined by observing spores and pre-developed hyphae of the fungus in contact with a solution of actinomycin (15  $\mu\text{g./ml.}$ ). Inhibition of spore germination, and swelling, distortion and excessive branching of the fungal hyphae identical with that reported for the actinomycetes E4 and E63 (Table 1) was again observed.

To study the effects in soil, known amounts of actinomycin (3, 5, 10, 15  $\mu\text{g./g.}$  soil) were mixed with the sterile neutral soil before embedding slides containing spores and pre-developed hyphae. The typical antibiotic effects were again noted with all concentrations of actinomycin used. In view of these effects, it is significant that antibiotic could not be recovered in extracts of these actinomycin-containing soils. Inasmuch as the *in vitro* and soil effects of actinomycin were identical with the effects of actinomycetes E4 and E63 under the same conditions, it would appear that the inhibitory and antagonistic action of these organisms in soil is, in fact, due to actinomycin.

## DISCUSSION

In a preliminary investigation of the antagonistic potential of a number of actinomycetes in soil (Stevenson, 1956) some evidence was produced which indicated that the competitive effects of these organisms were largely due to

specific antibiotic action. Thus there was a correlation between the degree of control of root-rot of wheat due to *Helminthosporium sativum* by a number of actinomycetes in pot experiments and the degree of antibiotic action shown by *in vitro* assay. But no relationship was detected between the effects of these actinomycetes in diminishing the disease and their ability to produce antibiotics in soil as assessed by soil-extraction techniques. In this present study, a direct observation technique was used to determine the effects of the actinomycete antibiotics of *Helminthosporium sativum*. Three additional lines of evidence support the theory of specific antibiotic action in soil. This new evidence depends on: (1) on a correlation between the degree of inhibition of spore germination by the eight actinomycetes in soil and *in vitro*; (2) the close resemblance between the specific effects produced by these actinomycetes on the hyphae of *H. sativum* in soil and those produced by the actinomycete antibiotics *in vitro*; (3) the fact that the very characteristic effects caused by *Streptomyces antibioticus* resemble exactly those brought about in soil and *in vitro* by actinomycin which is produced by this actinomycete. In addition, antibiotic activity in actinomycete-inoculated unsupplemented soils has also been demonstrated.

Although most of these studies were carried out in sterile soils, it is significant that the antibiotic effects of the actinomycetes observed on *Helminthosporium sativum* are very similar to those detected when this fungus is placed in natural soils. In view of the much higher concentration of antagonistic organisms in such soils, a more rapid rate of destruction is to be expected. Nevertheless, such typical antibiotic effects as inhibition of germination, hyphal distortion, and lysis, are readily recognizable in these non-sterile soils.

The frequent failure to extract detectable quantities of antibiotics from natural soils does not preclude the presence and activity of these substances in them. It is possible that antibiotic production occurs only in restricted loci, such as, in the vicinity of local concentrations of suitable carbon sources. Under such conditions, a localized antibiotic effect might well be exerted on susceptible organisms even though the overall concentration of antibiotic material is too small to be detected by extraction. For this reason it is felt that direct observation techniques are necessary for the detection of antibiotic activity in such physically restricted environments.

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## EXPLANATION OF PLATES

## PLATE 1

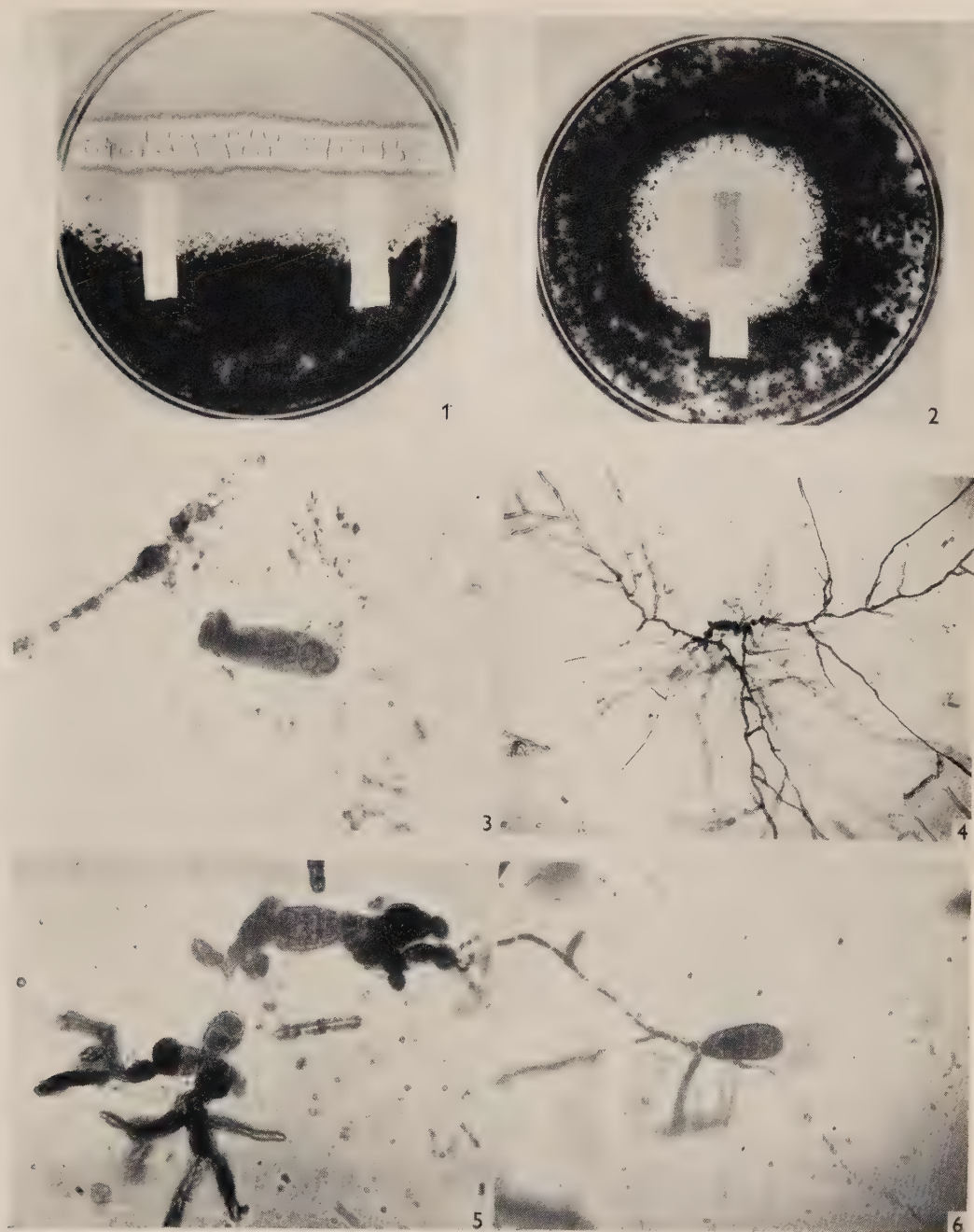
- Fig. 1. Plate technique used for assessing the antibiotic effects of actinomycetes on spores of *Helminthosporium sativum*.
- Fig. 2. Plate technique used for assessing the antibiotic effects of actinomycetes on pre-developed hyphae of *H. sativum*.
- Fig. 3. Spore of *H. sativum* showing post emergence inhibition in the presence of the antibiotic of actinomycete AA24 ( $\times 343$ ).
- Fig. 4. Hyphal distortion in the presence of the antibiotic of actinomycete AA27 ( $\times 77$ ).
- Fig. 5. Swollen, distorted and branched appearance of *H. sativum* in the presence of the antibiotics of actinomycetes E4 and E63 ( $\times 343$ ).
- Fig. 6. Lysis of pre-developed hyphae of *H. sativum* in soil inoculated with actinomycete AA24 ( $\times 343$ ).

## PLATE 2

- Fig. 7. Post-emergence inhibition in soil inoculated with actinomycete E63 ( $\times 392$ ).
- Fig. 8. Distortion and lysis of *H. sativum* in soil inoculated with actinomycete E4 ( $\times 392$ ).
- Fig. 9. Typical hyphal protuberances formed by *H. sativum* in the presence of the actinomycete XX19 in soil; similar to the distortion noted in the *in vitro* studies ( $\times 88$ ).

(Received 25 April 1956)





I. L. STEVENSON—ANTIBIOTIC ACTIVITY OF ACTINOMYCETES IN SOIL. PLATE 1  
(Facing p. 380)



I. L. STEVENSON—ANTIBIOTIC ACTIVITY OF ACTINOMYCETES IN SOIL. PLATE 2

FETNER, R. H. & INGOLS, R. S. (1956). *J. gen. Microbiol.* **15**, 381-385

## A Comparison of the Bactericidal Activity of Ozone and Chlorine against *Escherichia coli* at 1°

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**SUMMARY:** The bactericidal effects of ozone solutions were tested against *Escherichia coli* suspensions at 1°, and the lethal concentration was found to be that quantity of ozone necessary to produce a detectable residue in the suspension; under the conditions of our experiments this was 0.4-0.5 mg./l. A comparison of the bactericidal activity of chlorine under similar conditions emphasized the different modes of action of the two agents.

Quantitative studies on the antibacterial activity of ozone are difficult because of the instability of ozone solutions. The decomposition of ozone in solution is described as occurring in a stepwise fashion, producing in turn hydroperoxyl and hydroxyl radicles (Adler & Hill, 1950), both of which exhibit considerable biological activity. The decomposition process is temperature dependent and is catalysed by hydroxyl ion (Adler & Hill, 1950; Stumm, 1954). The usual process of bubbling ozone through a bacterial suspension and then measuring the total oxidizing residue iodometrically fails to give an accurate determination of the amount of ozone present. Adler & Hill (1950) pointed out, for example, that KI solutions measure the total oxidizable capacity rather than the amount of ozone only. It was deemed necessary that before biological investigations, a study should be made on the chemistry and kinetics of ozone solutions and an evaluation made on the analytical techniques of ozone determination under conditions appropriate to this problem. The detailed results of these studies were presented elsewhere (Ingols, Fetner & Eberhardt, 1956); it was found that ozone solutions at 1° and pH 2 (0.01 N-H<sub>2</sub>SO<sub>4</sub>) are relatively stable, showing little decomposition over a period of 8 hr. The analytical method of choice for ozone determination in such solutions is the ferrous ion method, in which ferrous ion (as Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O) is oxidized to the ferric state and then the excess of ferrous ion titrated with potassium permanganate. It was decided that by treating bacterial suspensions with dilutions of such stable solutions of ozone at (1° and pH 2), a more definite interpretation could be made of the activity of ozone itself. To obtain additional information about ozone activity at the concentrations of greatest biological interest, oxidation-reduction (O/R) potentials of buffered bacterial suspension were determined after the addition of various amounts of ozone.

### METHODS

The organisms (strain 'B' obtained from Professor H. Wycoff, a representative strain which behaved biochemically as a typical member of *Escherichia coli*), were grown in nutrient broth (Difco) at 37° for 18 hr. Test suspensions

were diluted to contain *c.*  $5 \times 10^4$  viable organisms/ml. in 100 ml. of 0.01M-phosphate buffer (pH 6.8), chilled to 1° (30–45 min.), and treated with ozone or chlorine (Zonite) from stock solutions. After the desired period of contact the reaction was stopped by the addition of excess sodium thiosulphate (5.0 ml. 0.1N) the solutions returned to room temperature and plate counts made according to standard procedures (American Public Health Association, 1955). The ozone was generated in a laboratory ozonator (Welsbach Co.; Philadelphia; Model T-23) using commercial oxygen as the gas supply. The ozone+oxygen mixture was bubbled through (0.1N-H<sub>2</sub>SO<sub>4</sub>) solution in a gas scrubber. The ozonator, oxygen gas cylinder and the water cooler for the ozonator were located in a refrigerated room where all the low-temperature work was performed. Glass-distilled and de-ionized water (specific conductance at 25° =  $0.94 \times 10^{-6}$  reciprocal ohms) was used in all experimental work.

The ferrous-ferric ion analytical technique (Ingols *et al.* 1956) was used to analyse the ozone concentration of the stock solution. Enough ferrous ammonium sulphate was added to measured samples of the ozone solution to bring 100 ml. of it to *c.* 0.005N and the non-oxidized ferrous ion was then titrated with 0.1N-potassium permanganate. Chlorine concentrations were determined iodometrically according to the methods prescribed by the American Public Health Association (1955).

Oxidation-reduction potential measurements were made with a platinum-calomel electrode system connected to a Beckmann (model H-2) pH meter. In these experiments buffered bacterial suspensions were used which were similar to those in the dose/contact time experiments. To these bacterial suspensions, successive equal volumes of an ozone solution were added at 1°, and the O/R potential (mV) measured.

## RESULTS

Table 1 summarizes the information obtained on the bacterial properties of ozone. This information reveals that: (1) The bactericidal concentration of ozone for the *Escherichia coli* strain used under these experimental conditions (<1% survivors) was between 0.4 and 0.5 mg./l.; (2) the concentration of ozone necessary to effect this kill had a very critical value; (3) at this con-

Table 1. *Survival of Escherichia coli in different concentrations of ozone in solution after various contact times at 1°*

Initial O <sub>3</sub> concentration (mg./l.)	Period of exposure (min.)					
	1	2	4	8	16	32
	Survival (%)					
1.00	<1	<1	<1	<1	—	—
0.75	<1	<1	<1	<1	<1	<1
0.63	<1	<1	<1	<1	<1	<1
0.53	<1	<1	<1	<1	<1	<1
0.42	75	<1	71	<1	73	46
0.31	96	97	74	70	92	—
0.21	33	82	99	99	79	69



centration, a contact time greater than 1 min. was not necessary; (4) at ozone concentrations near or just smaller than lethal, the bactericidal action was irregular, probably an expression of the instability of ozone solutions.

The results of the chlorination studies are given in Table 2, which indicates that chlorine (as  $\text{Cl}_2$ ) at a concentration of 0.25–0.30 mg./l. was effective at 1° and pH 6.8 in decreasing the bacterial count to 1% of the control in 1 to 10 min.

Table 2. *Survival of Escherichia coli in different concentrations of chlorine in solution at 1° after various contact times*

Initial concentration of chlorine (mg./l.)	Period of exposure (min.)						
	1	2	4	8	16	32	64
	Survival (%)						
1.10	<1	<1	<1	<1	<1	<1	<1
0.52	<1	<1	<1	<1	<1	<1	<1
0.35	1.5	<1	<1	<1	<1	<1	<1
0.27	2.0	1.5	1.6	1.2	1.0	<1	<1
0.25	24	19	12	13	8	7	5
0.17	70	53	39	24	15	4	<1
0.10	87	66	64	46	46	45	40

Fig. 1 emphasizes the difference between the bactericidal activities of ozone and chlorine. When the surviving fraction of bacteria is plotted against concentration, with a common period of exposure to the bactericidal agents, the chlorination results show a typical logarithmic curve, whereas ozone produced an immediate complete kill at the critical concentration.

To obtain additional information about ozone activity at the concentrations of greatest biological interest, oxidation-reduction (O/R) potentials of buffered bacterial suspensions were determined after the addition of various amounts of ozone. It was reasoned that the O/R potential at or close to the lethal concentration would exhibit a demonstrable change indicative of the corresponding activity. Fig. 2 presents the results of the experiment; it can be seen that there was a sharp break in the O/R potential at an ozone concentration comparable to the lethal concentration in the dosage contact time experiments. A differential plot of the same data emphasizes this information (Fig. 3).

#### DISCUSSION

The lethal concentration of ozone for the strain of *Escherichia coli* (0.4–0.5 mg./l.) under these experimental conditions is higher than the values obtained by other workers (Bringmann, 1954; Leiguarda, Peso & Polazzolo, 1949), who determined the ozone residue as total oxidizable constituents remaining after bubbling ozone through a test solution. This slightly higher ozone value may be the result of temperature dependency or of our use of the dose required rather than residue accumulated. Each particular test solution can be assumed to have a certain ozone consumption.

A comparison of the bactericidal activity of ozone and chlorine emphasizes the different modes of action of the two agents. The surviving fraction of chlorine-treated bacteria decreases in geometric progression as a function of time or concentration (Fig. 1). Ozone, on the other hand, showed an all-or-

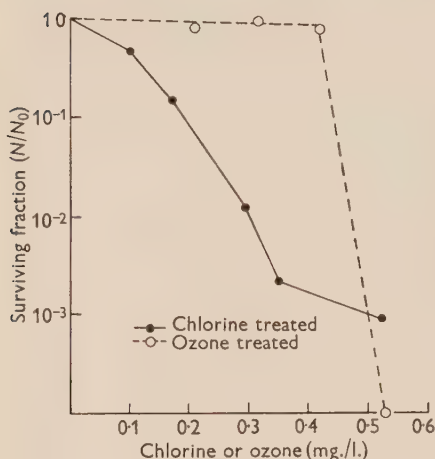


Fig. 1. Surviving fraction of *Escherichia coli* as a function of chlorine or ozone concentration; contact time 16 min. at 1°.

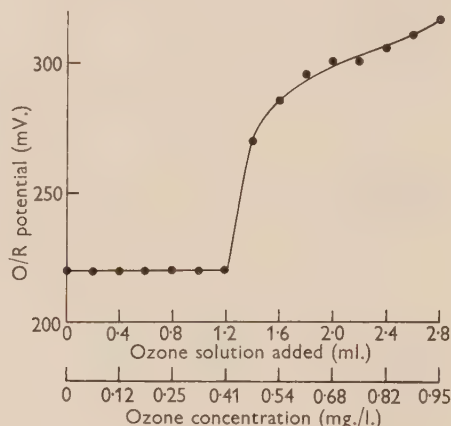


Fig. 2. Oxidation-reduction potential of suspensions with addition of various amounts of ozone solution. (*Escherichia coli* buffered suspension at 1° and pH 6.8.)

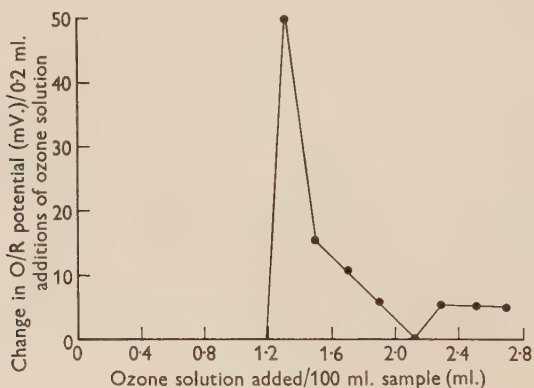


Fig. 3. Differential plot of data of Fig. 2.

none type of effect within the contact of 1 min.; there was no effect of the ozone below a certain critical concentration and above this concentration there were no detectable survivors. We have determined that this critical concentration represents that quantity of ozone necessary to produce a detectable residue in suspension. This information tends to support the theory of Bringmann (1954) that ozone acts as a general protoplasmic oxidant.

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## Nutritional Studies with the *Clostridium botulinum* Group

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**SUMMARY:** The factors which influence the initiation of growth from small inocula of *Clostridium parabolulinum* type A in the defined medium previously described were re-examined. CO<sub>2</sub> was found essential for rapid and regular growth from small inocula; it could be partially replaced under conditions of strict CO<sub>2</sub> depletion by an unidentified factor present in enzymic hydrolysate of casein or yeast extract.

The growth requirements of the various serological types of *C. botulinum* group were studied and compared with those of *C. parabolulinum* type A. It was found that the various organisms can be classed into two nutritional groups: (a) those which gave rapid and heavy growth in a chemically defined medium as described for type A, with some variations in respect of vitamin requirements: here belong *C. parabolulinum* type A (12 strains tested), type B (9 strains) and *C. sporogenes* (10 strains); (b) organisms which failed to grow in the defined medium or in a partially-defined medium (containing acid hydrolysate of casein) supplemented with all known B-vitamins: this group comprised organisms of the type C (2 strains) type D (4 strains) and type E (5 strains, of which 3 grew in a partially defined medium).

The similarity in nutritional requirements of *Clostridium sporogenes* to *C. parabolulinum* types A and B and the similarity in cultural and biochemical properties make *C. sporogenes* indistinguishable from, and perhaps identical with, non-toxicogenic strains of *C. parabolulinum* types A and B.

In a previous communication (Mager, Kindler & Grossowicz, 1954) the nutritional requirements of *Clostridium parabolulinum* type A were described. The present paper deals with factors which determine the growth of *C. parabolulinum* type A from small inocula. In addition, the growth requirements of other *C. botulinum* types and *C. sporogenes* were studied and compared with those of *C. parabolulinum* type A.

### METHODS

The general procedure and the growth assays were carried out as described (Mager *et al.* 1954). Mercaptoacetate (0.05 %) and cysteine (0.025 %) were present as reducing agents.

*Organisms.* The 43 strains used in this study were obtained from the following sources: National Collection of Type Cultures (NCTC) England (11 strains); American Type Culture Collection (ATCC) U.S.A. (16 strains); U.S. Depart-

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ment of Agriculture (7 strains); N.Y. Department of Health (NYDH) (3 strains); Department of Bacteriology, the Hebrew University, Jerusalem (1 strain); Dr K. F. Meyer, University of California (3 strains); Dr D. A. Boroff, State Research Hospital, Galesburg, Ill. (1 strain), Dr J. Keppie, Microbiological Research Department, Porton, Wiltshire, England (1 strain).

## RESULTS

*Effect of CO<sub>2</sub> on growth of Clostridium parabolulinum type A*

In the described medium excellent growth was obtained regularly with large inocula (0.1 ml. of 24–48 hr. culture containing *c.*  $0.5 \times 10^8$  organisms). On the other hand, growth was irregular and the lag period was very much prolonged when smaller inocula were used ( $1 \times 10^5$  organisms or less). It therefore became apparent that some factor is required for growth initiation. Since it was shown by Gladstone, Fildes & Richardson (1935), with a rigid technique, that CO<sub>2</sub> exerts a controlling effect on the initiation of growth of both aerobic and anaerobic bacteria, the effect of CO<sub>2</sub> was tested. The medium was acidified to pH 2, boiled for 10–15 min. to remove CO<sub>2</sub> and the pH value readjusted to 7.4 with freshly distilled ammonia solution. After autoclaving, the medium was rapidly cooled, inoculated, and incubated anaerobically in a McIntosh and Fildes jar. Under these conditions no growth, or irregular growth, was obtained when small inocula were used, whereas with large inocula growth was unimpaired. This was in contrast to the results obtained in a medium which contained an enzymic casein hydrolysate where no adverse effect of CO<sub>2</sub>-removal by acidification etc., was noticed (Table 1). Since acidification (in order to remove bound CO<sub>2</sub>) of the enzymic casein hydrolysate medium (ECH

Table 1. *Effect of size of inoculum on growth of Clostridium parabolulinum type A in CO<sub>2</sub>-free medium*

The partially-defined medium contained (mg./10 ml.): glucose 50; Casamino acids vitamin-free (Difco) 50; L-tryptophan, 0.05; L-arginine 30; DL-phenylalanine 20; L-tyrosine 2.5; L-cysteine 2.5; Na-mercaptoacetate 5. Salts A 0.1 ml. and salts B 0.01 ml. (Snell, Strong & Peterson 1937). Biotin, 0.005 µg; thiamine 4 µg; *p*-aminobenzoic acid, 0.1 µg. The ECH medium was the partially-defined medium (as above) supplemented with 0.5% Enzymic Casein Hydrolysate (Difco).

Dilution of inoculum (undiluted = <i>c.</i> $5 \times 10^8$ organisms)	Relative degree of growth after 24 hr. (% transmission)	
	Partially defined medium (%)	ECH medium (%)
10 <sup>-1</sup>	18	17
10 <sup>-2</sup>	18	17
10 <sup>-3</sup>	35	18
10 <sup>-4</sup>	80	18
10 <sup>-5</sup>	95	18
10 <sup>-6</sup>	100	18
10 <sup>-7</sup>	100	20
10 <sup>-8</sup>	100	25

medium) did not alter the results, it became obvious that the enzymic casein hydrolysate contained material capable of replacing  $\text{CO}_2$ .

In order to determine the nature of the  $\text{CO}_2$  replacing factor (CRF) a number of substances known as products of  $\text{CO}_2$  fixation were tried. The following compounds proved active: nucleic acids, guanine, xanthine and adenine (Table 2).

Table 2. *Effect of various substances on growth of Clostridium parabotulinum from small inocula in a  $\text{CO}_2$ -deficient medium*

$\text{CO}_2$  was removed by evacuation of the McIntosh and Fildes jar the traces of residual  $\text{CO}_2$  being absorbed with concentrated KOH. The inoculum was  $5 \times 10^2$  organisms of *C. parabotulinum* type A strain 1.

Substances added to the partially defined medium (Table 1) (mg./10 ml.)		CO <sub>2</sub> removed by			
		Evacuation		Evacuation + KOH	
		Period of growth (hr.)			
		24	72	24	72
		Relative degree of growth (% transmission)			
No addition		100	95	100	100
Sodium bicarbonate*	2.0	20	80†	20	65†
Enzymic casein hydrolysate (Difco)	50.0	18	85	18	90
Enzymic casein hydrolysate (Difco)	10.0	30	40	63	32
Enzymic casein vitamin-free	50.0	76	22	100	88
Yeast extract (Difco)	50.0	18	72	17	65
Ribonucleic acid	1.0	75	18	100	100
Deoxyribonucleic acid	1.0	80	17	100	100
Guanine	0.1	76	20	100	100
Xanthine	0.1	78	18	100	100
Adenine	0.1	75	19	100	100
Uracil	0.1	100	96	100	100
Thymine	0.1	100	95	100	100
Hypoxanthine	0.1	100	98	100	100
Malate*	50.0	100	92	100	100
Pyruvate*	50.0	100	96	100	100
Citrate	50.0	100	95	100	100
Acetate	50.0	100	95	100	100
Formate	50.0	100	93	100	100
Succinate	50.0	100	97	100	100
α-Keto-glutarate*	50.0	100	92	100	100
Oxalacetate*	50.0	70	20	45	25

\* Sterilized by Seitz filtration and added aseptically to the partially defined medium.

† The decrease of turbidity on continued incubation was due to autolysis.

Among the Krebs-cycle intermediates only oxalacetate possessed CRF-activity. However, when traces of  $\text{CO}_2$  were removed from the jar by concentrated KOH, only bicarbonate, oxalacetate, enzymic casein hydrolysate and yeast extract enabled growth from small inocula to occur (Table 2). It cannot be ascertained, however, whether the effect of oxalacetate may not have been due to  $\text{CO}_2$  released by spontaneous decarboxylation.

*Sources and properties of the CRF.* Besides casein hydrolysate good sources of CRF were yeast extract (Difco), proteose-peptone (Difco) and a Seitz-filtrate of an *Escherichia coli* culture grown in a casein hydrolysate (hydrolysed with acid) medium. The active substance of the enzymic casein hydrolysate was heat stable and was not destroyed by heating for 1 hr. at 120° in 5N-HCl or N-NaOH. The CRF was removed from the enzymic casein hydrolysate by repeated treatment with Norite A at pH 3.0; some CRF activity was obtained by continuous extraction with ethyl acetate or acetone.

*Stimulatory effect of non-essential amino acids.* The rapid growth regularly obtained in the presence of CO<sub>2</sub> allowed re-evaluation of the importance for growth of the non-essential amino acids when relatively small inocula were employed. With an inoculum of  $1 \times 10^5$  organisms, maximal growth was obtained after 16–24 hr. of incubation in a medium composed of a mixture of 19 amino acids (see Table 3).

Table 3. *Effect of threonine, histidine, glycine and serine on growth of Clostridium parabolinum type A*

Hall strain (ATCC 3502): inoculum,  $1 \times 10^5$  cells.

	Period of growth (hr.)			
	17	24	41	65
	Relative degree of growth (% transmission)			
19 amino acids*	41	35	32	43
8 amino acids†	95	90	87	82
8 amino acids + glycine + histidine	87	34	36	67
8 amino acids + serine + histidine	84	46	47	50

\* Nineteen amino acids in defined medium (Mager *et al.* 1954).

† Eight amino acids: cysteine + the following 7 essential amino acids: L-arginine, DL-isoleucine, DL-leucine, DL-methionine, DL-phenylalanine, L-tryptophan and L-tyrosine.

In a medium containing the 8 essential amino acids (L-arginine, DL-isoleucine, DL-leucine, DL-methionine, DL-phenylalanine, DL-threonine, L-tryptophan, L-tyrosine) and cysteine as a reducing agent, rapid growth was obtained with a large inoculum ( $5 \times 10^5$  organisms). However, with a small inoculum ( $1 \times 10^5$  organisms) no growth appeared at all or it was delayed for 3–4 days. This lag was substantially decreased by the addition of glycine and histidine to the above mixture of essential amino acids. Addition of glycine abolished the requirement for threonine, glycine becoming essential in the threonineless medium; serine replaced glycine. These findings are illustrated in Table 3 with 1 out of the 6 strains tested. Among the various strains of type A, some preferred glycine whereas others responded better to serine.

As reported previously, the demand for excessive amounts of arginine in a casein hydrolysate medium could be satisfied partially by ornithine and lysine (Mager *et al.* 1954). In an arginine-free medium, however, neither ornithine nor lysine was able to support growth. Thus, ornithine and lysine showed a sparing effect on arginine requirement. Citrulline, on the other hand, was as effective as arginine in promoting growth.

*Nutrition of Clostridium parabolulinum type B*

All the 8 strains tested grew in the defined medium (19 amino acids, vitamins, salts), which was found adequate for cultivation of *Clostridium parabolulinum* type A. A closer comparison in regard to vitamin and amino acid requirements did not reveal any significant differences between type B and type A strains. In respect of vitamin requirements the same pattern of variability was found as with type A strains. Thus, all the type B strains required biotin and *p*-aminobenzoic acid (*p*-AB). Two out of 8 strains showed a dependence on pyridoxine or pyridoxamine. Thiamine was stimulatory for some strains.

In general the rate of growth of type B strains was somewhat slower than that of type A strains; however, no differences in either physico-chemical (optimal pH value and temperature) or metabolic properties (utilization of glucose, salicin etc.) were found.

*Nutrition of Clostridium sporogenes*

*Clostridium sporogenes* appears to be closely related to *C. parabolulinum* type A in cultural, biochemical and serological properties (Hadley, 1927). The nutritional requirements of this organism were studied by Knight & Fildes (1933), Fildes & Richardson (1935), and Shull, Thoma & Peterson (1949). The later investigators established both the vitamin and amino acid requirements of this organism but made no distinction between essential and stimulatory amino acids.

In view of the observed interplay between non-essential and essential amino acids in *Clostridium parabolulinum* type A (see above) the possibility of distinguishing between *C. parabolulinum* and *C. sporogenes* by a study of amino acid interrelationships was explored. All the 10 strains tested grew well in the defined medium (Mager *et al.* 1954). The same 8 amino acids were found to be essential for *C. sporogenes* and for *C. parabolulinum* types A and B. Moreover, serine or glycine was able to replace threonine. In respect of arginine requirement, the strains of both species behaved alike (Shull *et al.* 1949). All the strains of *C. sporogenes* required biotin and *p*-AB whilst nicotinic acid and thiamine were found stimulatory each for one strain only (strains NTCC 534 and ATCC 7955 respectively). Similarly, no biochemical differences were found which would aid in distinguishing non-toxicogenic strains of *C. parabolulinum* from *C. sporogenes*.

*Nutrition of Clostridium botulinum types C, D and E*

*Type C strains.* Two toxigenic and two non-toxicogenic strains were examined. The two non-toxicogenic strains were successfully cultivated in the same chemically defined medium as that described for types A and B. These organisms, however, showed typical R-appearance (rhizoid colonies on agar and clumping in liquid media) and were devoid of toxicity for mice and chickens and therefore indistinguishable from *C. sporogenes*. Hence it seems doubtful whether



these strains are true varieties of the C group as labelled (strains 462 and 6060 of the ATCC). The toxigenic strains (one from Dr K. F. Meyer and the other from Dr D. A. Boroff) did not grow in the defined or partially-defined media, even on enrichment with a mixture of all the known B vitamins (see legend to Table 4.)

Table 4. *Vitamin requirements of Clostridium botulinum type E*

Basal medium (mg./ml.); Casamino acids vitamin-free (Difco), 100; glucose, 50; L-tryptophan, 0.5; L-cysteine, 2.5; Na mercaptoacetate, 5; Na bicarbonate, 2. Salts A, B (Snell *et al.* 1937) 0.1 ml. and 0.01 ml./10 ml. medium, respectively.

Strains* (no.)	Vitamins added ( $\mu$ g.) to 10 ml. basal medium	Period of growth (hr.)	
		24	48
		Relative degree of growth (% transmission)	
1, 2	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxamine 0.05	69	30
	Biotin 0.005, thiamine 4, folic acid 0.1	100	100
	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxine 5	95	90
	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxine 500	67	39
	— thiamine 4, folic acid 0.1, pyridoxamine 0.05	100	100
	Biotin 0.005, — folic acid 0.1, pyridoxamine 0.05	95	90
	Biotin 0.005, thiamine 4, — pyridoxamine 0.05	100	100
	Biotin 0.005, thiamine 4, <i>p</i> -AB 0.1, pyridoxamine 0.05	100	100
	Biotin 0.005, thiamine 4, CF 0.01, pyridoxamine 0.05	70	29
	Biotin 0.005, thiamine 4, folic acid 0.1, pyridoxamine 0.05, ECH 0.1 %	23	15
3	Pantothenic acid 10, nicotinic acid 10	42	48
	Pantothenic acid 10	95	90
	Nicotinic acid 10	92	95
	Pantothenic acid 10, nicotinic acid 10, ECH† 0.1 %	16	20
4, 5	Mixture of 19 growth factors‡	100	100

\* Strain 1=ATCC 9565; strain 2=NYDH 36208; strain 3=NCTC 8266; strain 4=ATCC 9564 and strain 5=obtained through the courtesy of Dr K. F. Meyer.

† ECH=enzymic casein hydrolysate (Difco).

‡ Nineteen growth factors ( $\mu$ g./10 ml.): biotin, 0.005; thiamine, 4; folic acid, 0.1; *p*-aminobenzoic acid (*p*-AB) 0.1; citrovorum factor (CF), 0.1; choline, 25; Ca pantothenate, 10; pyridoxine 5; pyridoxamine 5; pyridoxal HCl 5; pyridoxal phosphate 5; nicotinic acid, 10; nicotinamide, 10; riboflavin, 5; inositol, 400; vitamin B<sub>12</sub>, 0.5; biocytin, 0.1;  $\alpha$ -lipoic acid, 0.01.

*Type D strains.* Three strains were tested: none grew in the defined medium or in the medium enriched with all the known B vitamins.

*Type E strains.* Five strains were studied; all were toxigenic, but the toxic culture filtrate (growth in cooked meat broth) was of low titre. Two strains (ATCC 9564 and another obtained through the courtesy of Dr K. F. Meyer) did not grow in the defined or in the partially-defined media even on supplementation with various accessory growth factors (Table 4). However, addition of enzymic casein hydrolysate produced heavy growth of these strains.

The remaining 3 strains gave luxuriant growth in the partially-defined medium. One of them (NCTC 8266) required pantothenic and nicotinic acids,

being the only one out of 43 strains of *Clostridium botulinum* tested which did not need biotin for growth. The other 2 strains of this group (ATCC 9565 and NYDH 36208) required biotin, thiamine, pyridoxamine and folic acid or citrovorum factor. Folic acid could not be replaced by *p*-aminobenzoic acid. On the other hand, pyridoxal was as effective as pyridoxamine, whilst pyridoxine was much less effective (c. 0.1 %). The results are summarized in Table 4.

Replacement of the acid hydrolysate of casein in the partially defined medium by a mixture of 19 amino acids resulted in only slightly inferior growth. The essentiality of different amino acids however was not determined.

## DISCUSSION

Like many other micro-organisms (Fildes & Richardson, 1935) the parabolulinum group exhibits dependence on a CO<sub>2</sub> supply for the initiation of growth. The growth-initiating effect of CO<sub>2</sub> is not yet fully understood. CO<sub>2</sub> is known to be required for the synthesis of purines and various intermediates of the Krebs cycle (Pappenheimer & Hottle, 1940; Lwoff & Monod, 1947). However, none of these compounds, singly or in combination, was able to replace CO<sub>2</sub> when rigid conditions for CO<sub>2</sub>-removal were applied. Under conditions of CO<sub>2</sub> depletion an unidentified factor present in enzymic casein digests was able to replace CO<sub>2</sub>. This factor (CRF) is similar in properties to that required by *Escherichia coli* and *Aerobacter aerogenes* as described by Wong & Ajl (1953). Whether CRF is a product of CO<sub>2</sub> fixation or is a catalyst is not clear.

The distinction between essential and non-essential amino acids cannot always be rigidly drawn. Change of growth conditions, e.g. decreasing the size of inoculum, may necessitate the inclusion of an amino acid which is dispensable when a large inoculum is used. This possibility is exemplified by the requirement of *Clostridium parabolulinum* types A and B and *C. sporogenes*, for glycine (or serine) + histidine in a medium deficient in threonine. These interrelations suggest the existence of a biosynthetic pathway leading from serine or glycine to threonine. The interconversion between serine and glycine has been established in various micro-organisms (Sakami, 1955). Histidine may contribute to the synthesis of threonine, possibly by serving as a formyl (C-1) donor (Sprinson & Rittenberg, 1952; Toporek, Miller & Bale, 1952).

Summing up the experimental results it may be concluded that the various botulinum types fall into two nutritional groups: (a) strains which can be grown in a defined medium as described, notwithstanding differences in the vitamin requirements among the various strains; (b) strains which have a different nutritional pattern in respect of amino acids and of accessory growth factors. The first comprises strains of *Clostridium parabolulinum* types A and B. Evidently the serological and chemical differences between the two types of toxins are not reflected in the nutritional requirements of the organisms. The claim of Roessler & Brewer (1946) that *C. parabolulinum* type B differs from type A strains in requiring histidine instead of threonine, was not confirmed for the strains studied by us. *C. sporogenes* from a nutritional viewpoint appears indistinguishable from *C. parabolulinum* types A and B.

In view of the limited number of strains studied, it is difficult to decide to what extent the dispensability of thiamine (9 out of 10 strains of *Clostridium sporogenes* did not require thiamine) may serve as a guide for classifying a strain as *C. sporogenes* or as a non-toxicogenic variant of *C. parabotulinum*. However, all the strains of *C. sporogenes* may be classified tentatively at least together with *C. parabotulinum* types A and B in a 'parabotulinum group'.

This paper forms part of a Ph.D. Thesis presented by S. H. Kindler to the Hebrew University, Jerusalem. The helpfulness of the investigators mentioned in providing us with the various cultures is hereby gratefully acknowledged.

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## Toxin Production by *Clostridium parbotulinum* Type A

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**SUMMARY:** Factors which govern toxin production by *Clostridium parbotulinum* type A were studied in growing cultures and in non-proliferating cell suspensions. With growing cultures high toxin titres, comparable to those obtained in complex media, were obtained in a defined medium containing the following essential constituents: arginine, cysteine, histidine, isoleucine, leucine, methionine, phenylalanine, serine (or glycine), tyrosine, tryptophan, valine, glucose, vitamins, phosphate and magnesium. Most of the toxin was formed only after cessation of growth.

Resting suspensions of organisms produced toxin at a rapid rate; a three- to fivefold increase being obtained after 4 hr. incubation. It appears that the toxin is synthesized within the organisms and is liberated into the medium by autolysis. Release of toxin on artificial lysis by sonic oscillation, provided decisive evidence for this conclusion.

The effect of different metabolic inhibitors on toxin synthesis by resting organism suspensions was examined. Ethylenediamine tetracetic acid in amounts which did not inhibit growth suppressed completely toxin production. Some broad spectrum antibiotics (streptomycin, chloramphenicol, chlorotetracycline at 100 µg./ml.) inhibited toxin formation to about 50%. Penicillin did not affect toxin synthesis even at concentrations 1000-fold higher than those required to arrest growth of the organism.

The production of toxin by *Clostridium parbotulinum* type A has been studied hitherto in complex empirical media (Lewis & Hill, 1947; Stevenson, Helson & Reed, 1947). These studies revealed the importance of some factors which govern the accumulation of the toxic protein in the culture fluid; these included nutrients, temperature, pH value, and period of incubation. However, in view of the complexity of the media used, the chemical nature of the components required for toxin production could not be exactly determined. Following the development of a defined medium for the cultivation of *C. parbotulinum* type A (Mager, Kindler & Grossowicz, 1954), it became possible to study the production of toxin under defined conditions. The present communication describes toxin formation by proliferating cultures, and by resting organism suspensions, in defined media. The latter system permits a distinction between the requirements for multiplication and the factors which affect more directly toxin formation. The site of toxin synthesis (Nelson, 1927; Dack & Wood, 1928; Raynaud & Second, 1949), was also studied. A brief report has already been published (Kindler, Mager & Grossowicz, 1955).

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## METHODS

The general procedure, experimental conditions and the composition of the defined and partially defined media were described previously (Mager *et al.* 1954). Most experiments were performed with strains no. 1 (Hall, NCTC 3811) and no. 2 (Hebrew University, Jerusalem) of *Clostridium parbotulinum* type A. Stock cultures were kept in cooked meat broth. For the purpose of studying toxin production the organism was carried through several subcultures in the casein hydrolysate partially defined medium (CH., see legend to Table 1). This partially defined medium (10 ml.) was inoculated with 0.1 ml. of an 18–24 hr. culture.

*Composition of the complex (CCG) medium* (in %): Casein, 3; cornsteep liquor solids, 1; glucose, 0.5.

*Non-proliferating organisms* were prepared as follows: after 18 hr. growth in the casein hydrolysate partially-defined medium the organisms were collected by centrifugation (Servall centrifuge, 6000 r.p.m.) for 15–20 min. and washed with saline containing 0.05% mercaptoacetate, or with fresh medium as desired. The organisms were then resuspended in media of various compositions and incubated at 34° under anaerobic conditions.

*Extraction of toxin from the organisms.* *Clostridium parbotulinum* organisms were disintegrated by sonic oscillations by means of a Raytheon 9 KC Oscillator. The apparatus was cooled by circulating ice-cold water. Exposure of suspensions ( $2$  to  $6 \times 10^9$  organisms/ml.) for 15 min. to sonic vibrations resulted in destruction of most of the organisms (as revealed by decrease of turbidity and by microscopic examination). As shown by recovery experiments the titre of toxin filtrates, subjected to this treatment, remained practically unchanged.

*Determination of toxin titre.* Assays of toxin titre were carried out on white mice of 18–24 g. body weight by injecting intraperitoneally 0.5 ml. of toxin sample suitably diluted in a buffered gelatin medium (Lewis & Hill, 1947). The animals were kept at 28°, and the deaths recorded up to 4 days. The results were expressed in terms of MLD (minimal lethal dose) or LD 50 (Reed & Muench, 1938)/ml. of toxin sample.

## RESULTS

Previously we (Mager *et al.* 1954) reported that toxin of relatively low titre (c. 50,000 LD 50 doses/ml.) was obtained when *Clostridium parbotulinum* type A was grown in a defined medium. Subsequent experiments corroborated these results and also showed that on continued incubation in the defined medium the titre of the toxin declined much more rapidly than in complex culture media (Fig. 1). Since the amount of growth was about the same in the various media tested it seemed that the CCG medium provided factors which enhanced the formation of the toxin as well as its stability. A search for such factors was therefore conducted. As a first step, mixtures of various metabolites were added to the defined medium (e.g. nucleic acids and their components, vitamins of the B group, and members of the tricarboxylic acid cycle);

however, no increase of toxin titre was observed. Subsequently two important components of the complex medium, namely casein and cornsteep liquor (CSL) were added together or separately. The experiments showed that both ingredients increased the titre of the toxin, but most of the activity was due to the casein. In order to determine whether casein itself or some accompanying contaminant was responsible for the enhancement of toxin formation, purified casein (obtained by repeated precipitations with acetic acid and washing with ethanol and ether) was tested. As can be seen from Table 1, purified casein when added to the defined medium showed the same activity as commercial casein. Moreover, other proteins exhibited a similar effect. The question then arose whether degradation products would be as effective as the intact protein. Subsequent tests showed that addition to the defined medium of enzymic hydrolysates of casein or commercial peptones were effective in increasing the toxin titre, but did not prevent significantly the deterioration of the toxin formed. A similar effect was obtained by increasing the amino acid concentration of the medium (Table 1).

Table 1. *Effect of protein, protein hydrolysates and amino acids on the titre of botulinum toxin produced in various media mixtures*

Organism: *Clostridium parbotulinum* type A strain No. 2.

Media: CCG medium (in %): casein, 3.0; cornsteep liquor solids, 1.0; glucose, 0.5. CH medium (mg./10 ml.): Casamino acids vitamin-free (Difco), 50; L-tryptophan, 0.05; L-arginine, 30; DL-phenylalanine, 20; L-tyrosine, 2.5; Na-mercaptoacetate 5; salts A, B (Snell *et al.* 1937) 0.1 ml. and 0.01 ml./10 ml. respectively. The following substances were Seitz-filtered and added aseptically to the autoclaved medium (per 10 ml.): glucose, 50 mg.; L-cysteine, 2.5 mg.; NaHCO<sub>3</sub>, 2 mg.; biotin, 0.005 µg.; thiamine, 4 µg.; p-aminobenzoic acid, 0.1 µg.

Media + addenda	LD 50 dose/ml. after growth for	
	3 days	5 days
CCG	1,000,000	900,000
CH	100,000	10,000
CH + casein 3 %, cornsteep liquor 1 %	1,000,000	800,000
CH + cornsteep liquor 1 %	200,000	250,000
CH + casein 0.5 %	900,000	800,000
CH + casein (purified) 0.5 %	900,000	900,000
CH + edestin 0.5 %	700,000	600,000
CH + ovalbumin (crystalline) 0.5 %	750,000	800,000
CH + Bacto-peptone (Difco) 0.5 %	300,000	450,000
CH + Proteose-peptone (Difco) 0.5 %	500,000	500,000
CH + Casamino acids (Difco) 2 %	250,000	20,000
CH + tryptophan 0.5 %	400,000	50,000
CH + Casamino acids (Difco) 2 % + tryptophan 0.05 %	900,000	300,000

Since tryptophan was present only in small amounts in the defined medium, it was suspected of being the limiting factor. In fact, increasing the concentration of tryptophan (from 5 to 500 µg./ml.) resulted in a considerable increase of the toxin yield when tested after 2–3 days of incubation; on further incubation, however, a rapid decline of the toxin titre was observed (Fig. 1). Addition of protein to the tryptophan-enriched medium increased the stability of the toxin produced. The stabilizing effect of protein on the botulinum toxin was tested by incubation for 3 days of samples containing measured amounts of

crude toxin in the presence and in the absence of casein. The results, as presented in Table 2, show clearly the protective effect of the protein. Thus, at least two effects of protein were demonstrated: (a) increase of toxin formation, an effect which could be duplicated by increasing the concentration of tryptophan; (b) protection from deterioration (spontaneous) afforded by proteins but not by amino acids.

Table 2. *Influence of proteins on stability of botulinum toxin solutions*

	Toxin titre initial	LD 50/ml. after 3 days of incubation at 34°
Crude toxin preparation*	1,500,000	600,000
Crude toxin preparation + gelatin 0.5 %	1,500,000	1,000,000
Crude toxin preparation + casein 0.5 %	1,500,000	1,300,000
72 hr. culture†	750,000	200,000
72 hr. culture + casein (0.5 %) added after incubation for 24 hr.	800,000	700,000

\* Acid precipitate of whole culture (pH 3.5), eluted with phosphate buffer (pH 6.8).

† Grown in partially defined CH medium; strain no. 2.

*Resting organisms.* When *Clostridium paratubulinum* organisms are sown into a suitable medium, growth is characterized by a peak reached after 18–24 hr. of incubation, which is then followed by rapid decline and autolysis. Assays revealed small amounts of toxin during the logarithmic phase of growth; thereafter the titre increased rather steeply, reaching the maximum at the completion of lysis (Fig. 1). This sequence suggested that the toxin was apparently formed when stationary conditions prevail in the culture and multiplication therefore may not be an essential factor, and that toxin was released into the medium as a result of autolysis.

In order to study toxin production in the absence of multiplication, the technique of non-proliferating organisms was used (see Methods). Under these conditions the resting organisms, which were resuspended in the complete defined medium and incubated for 24 hr., formed large amounts of toxin. The toxin formed was proportional, within limits, to the density of the suspensions used (Fig. 2). Aerobic conditions were not detrimental to toxin formation by resting suspensions; practically the same yields of toxin were obtained under aerobic or anaerobic conditions.

In order to evaluate the importance of the various ingredients of the medium for toxin formation by non-proliferating organisms, the effect of omission of different components was tested. As shown in Table 3, the omission from the complete medium of either salts B, vitamins, or non-essential amino acids did not appreciably affect toxin formation. On the other hand, elimination of glucose, inorganic phosphates (salts A) or even one of the amino acids essential for growth (e.g. arginine) decreased the titre considerably. Especially interesting seemed the requirement for glucose. Non-proliferating organisms produced very little, if any, toxin when glucose was omitted from the medium. In growing cultures, omission of glucose resulted in about a ten-fold decrease in



toxin titre, while growth in the absence of glucose was decreased only by 50 % (Table 3).

*Site and kinetics of toxin production.* In non-proliferating organism suspensions, as in growing cultures, only small amounts of toxin were demon-

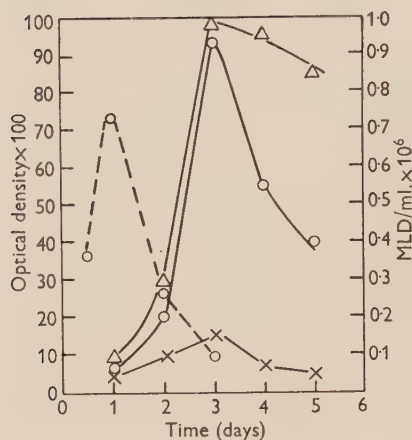


Fig. 1

Fig. 1. Growth and accumulation of toxin in various media. *Clostridium parbotulinum* type A (strain no. 1). ○---○, growth in defined medium; tryptophan 5 µg./ml. x—x, toxin formation in defined medium; tryptophan 5 µg./ml. ○—○, toxin formation in defined medium; tryptophan 500 µg./ml. △—△, toxin formation in defined medium + casein 0.5 %.

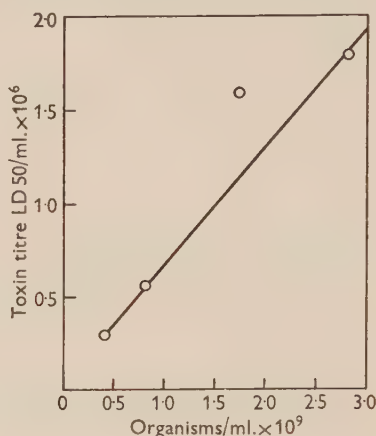


Fig. 2

Fig. 2. Toxin titre as a function of cell density. *Clostridium parbotulinum* type A, strain no. 1. Incubation time 17 hr. at 34°.

Table 3. *Effect of various nutrients on toxin production by non-proliferating suspensions of Clostridium parbotulinum type A, strain no. 2*

Incubation time: 24 hr. at 34°. Composition of incubation mixture (mg./10 ml.): DL-alanine, 5; DL-aspartic acid, 9; L-arginine, 30; L-cysteine, 2.5; DL-glutamic acid, 10; glycine, 10; L-histidine, 2; L-hydroxyproline, 1; DL-isoleucine, 5; DL-leucine, 15; L-lysine, 10; DL-methionine, 6; DL-phenylalanine, 20; L-proline, 4.5; DL-serine, 10; DL-threonine, 10; L-tryptophan, 5; L-tyrosine, 2.5; DL-valine, 20; glucose, 50; Na-mercaptoacetate, 5. Salt solutions (Snell *et al.* 1937) A and B, 0.5 ml. and 0.01 ml./10 ml. respectively. Vitamins (µg./10 ml.): biotin, 0.005; thiamine, 4; *p*-aminobenzoic acid, 0.1.

Substances omitted from the incubation mixture	MLD/ml. after 24 hr. incubation
None	2,500,000
Vitamins	2,500,000
Glucose	200,000
Salts A (phosphates)	300,000
Salts B (Mg <sup>++</sup> , Mn <sup>++</sup> , Fe <sup>+++</sup> , Na <sup>+</sup> )	1,750,000
Amino acids	100,000
Eight non-essential amino acids*	2,200,000
Tryptophan	150,000
Arginine	150,000
Valine	200,000
Threonine	2,200,000
Mercaptoacetate (aerobic conditions)	2,500,000

\* Alanine, aspartic acid, glutamic acid, hydroxyproline, lysine, proline, serine, threonine.



strated before noticeable lysis took place (Fig. 3). It appeared possible that the toxin molecule is synthesized within the organisms and is retained there until released by autolysis. In order to test this hypothesis disintegration of the organisms by sonic oscillation was used. Non-proliferating organisms were incubated in the complete medium (or with vitamins omitted), and samples were withdrawn at intervals; one portion was tested for toxicity without further treatment and another portion was centrifuged and the organisms,

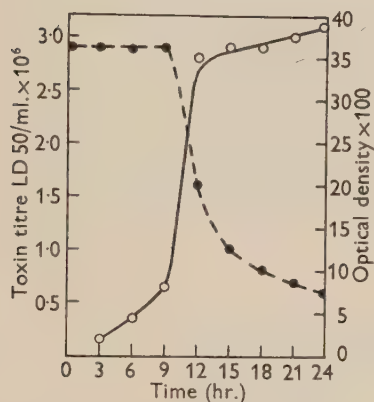


Fig. 3

Fig. 3. Correlation between autolysis and toxin production by non-proliferating cell suspensions of *Clostridium parbotulinum* type A (strain no. 2). ○—○, toxin titre; ●---●, turbidity of suspensions (measured after dilution 1/5). Initial concentration:  $4 \times 10^9$  organisms/ml. Incubation medium (in mg./10 ml.): casamino acids (Difco), 250, L-arginine, 30; DL-phenylalanine, 20; L-tryptophan, 5; L-tyrosine, 2.5; glucose, 50. Salts A (Snell *et al.* 1937), 1 ml. pH 7.2. Temperature of incubation 34°.

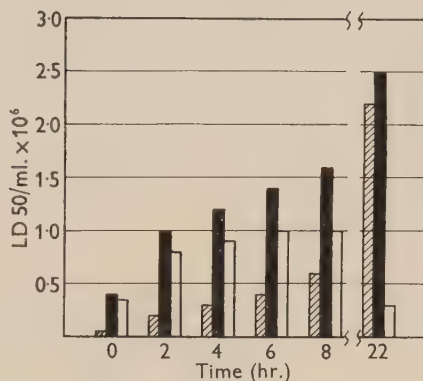


Fig. 4

Fig. 4. Amounts of free and bound toxin as a function of incubation time. Suspension concentration:  $3 \times 10^9$  organisms/ml. of *Clostridium parbotulinum* type A, strain no. 2. Incubation medium—as in Fig. 3. ▨, free toxin (amount of toxin in the medium before sonic disruption); ■, total toxin (amount of toxin in the medium after disruption of organisms by sonic oscillations); □, cell-bound toxin (total toxin—free toxin).

after resuspension, were subjected to sonic disruption. Fig. 4 shows that appreciable amounts of toxin were found within washed organisms (toxin assayed after sonic disruption) immediately after resuspension (zero time). In the course of incubation, the amount of intracellular toxin rapidly increased and after 4 hr. a threefold increase was obtained, amounting to about 50% of the final amount of toxin obtained after 22 hr. of incubation. In other experiments (Kindler, Mager & Grossowicz, 1955) even an eightfold increase was reached during a similar incubation period. The amount of 'free toxin' (spontaneously released into the medium) found in the untreated samples was only a small fraction of the total (i.e. cell-bound toxin+free toxin). On continued incubation (12–18 hr.) autolysis became pronounced and most of the toxin was set free. At this stage, as would be expected, sonic treatment increased only slightly the total yield of the toxin.

*Toxin formation by cell-free preparations.* Freshly harvested organisms were disrupted by sonic oscillation, the lysate added to the medium (as described for

resting organisms) and incubated for 24 hr. at 34°. The growth of organisms which might have survived the sonic treatment, was eliminated by adding penicillin (10 units/ml.). Synthesis of toxin was not demonstrated under these conditions.

*Inhibition of the toxin synthesis.* In the hope of gaining more insight into the mechanism of toxin synthesis the effect of substances likely to interfere with biosynthetic processes was tested. The substances tested included various antibiotics, respiratory poisons and chelating agents. As can be seen from Table 4, addition of penicillin, even in amounts 1000-fold greater than that necessary for growth inhibition, had no effect on the toxin synthesis; no penicillinase was found in the suspension of organisms. However, streptomycin chloramphenicol, oxytetracycline and chlortetracycline at 100  $\mu$ g./ml. inhibited toxin formation by about 50 %; growth was completely inhibited at 10  $\mu$ g./ml. Other known metabolic inhibitors (arsenate, arsenite, dinitrophenol, azide) suppressed both growth and toxin synthesis at nearly the same concentrations.

Table 4. *Effect of different compounds on growth and toxin production by Clostridium parbotulinum type A, strain no. 2*

The growth medium was the CH medium (see Table 1).

Inhibitor added		Percentage inhibition of	
		Growth	Toxin production
Penicillin 1000 units/ml.		100	0
Streptomycin	100 ( $\mu$ g./ml.)	100	49
Chloramphenicol	100 ( $\mu$ g./ml.)	100	43
Chlortetracycline	100 ( $\mu$ g./ml.)	100	64
Oxytetracycline	100 ( $\mu$ g./ml.)	100	55
EDTA (versene)	1000 ( $\mu$ g./ml.)	100	100
EDTA (versene)	100 ( $\mu$ g./ml.)	0	90
EDTA (100 $\mu$ g./ml.) + Mg <sup>++</sup>	400 ( $\mu$ g./ml.)	0	10
Versene Fe-3 specific	100 ( $\mu$ g./ml.)	100	0
$\alpha$ , $\alpha$ -dipyridyl	50 ( $\mu$ g./ml.)	100	0
<i>o</i> -phenanthroline	10 ( $\mu$ g./ml.)	100	0
8-Hydroxyquinoline	50 ( $\mu$ g./ml.)	100	20
Na-arsenate	M/2,000	100	100
Na-arsenate	M/20,000	0	0
Na-arsenite	M/500	100	100
Na-arsenite	M/5,000	0	0
Na azide	M/2,000	0	0
2,4-Dinitrophenol	M/5,000	50	0
2,4-Dinitrophenol	M/200,000	0	0

Ethylenediamine tetracetate (EDTA) at 100  $\mu$ g./ml. suppressed almost completely toxin formation (80–100 %) without inhibiting growth. On the other hand, more specific iron complexing agents ( $\alpha$ ,  $\alpha$ -dipyridyl, *o*-phenanthroline and versene Fe-3 specific) had no effect on toxin synthesis at concentrations which completely inhibited growth. The effect of EDTA suggested that divalent cations may play a role in toxin synthesis. In fact, a mixture of divalent cations reversed completely the inhibition induced by EDTA. Further

screening revealed magnesium ion as the active agent. Omission of  $Mg^{++}$  (from an EDTA-free medium) did not result, however, in a marked depression of toxin synthesis.

#### DISCUSSION

The results presented show that resting *Clostridium parbotulinum* type A synthesizes considerable amounts of toxin in a short period of time (4–8 hr.). Thus toxin formation can be studied independently of bacterial multiplication. Consideration may be given to the site of toxin formation. The fact that large amounts of toxin can be released from washed organisms by sonic disintegration provides evidence for the intracellular formation of the toxin. It appears, therefore, that accumulation of the toxin in the culture depends on an increased cell permeability of the organisms or autolysis. In this connexion it should be noted that only the extracellular ('free') toxin is effective *in vivo*, whilst washed intact organisms (containing large amounts of 'bound' toxin) are practically non-toxic, although the amount of bound toxin present in the organisms may exceed the lethal dose by a factor of 5–10 (see, for instance, Fig. 4).

The following possibilities were considered to explain this phenomenon. (a) Sonic treatment activates the 'cell-bound' toxin by depolymerization (see Boroff, Raynaud & Prévot, 1952; Wagman & Bateman, 1953) or by some other alteration of the toxin molecule. (b) The 'bound' toxin forms, with some cell constituent, an inactive complex which may be broken up by autolysis or, artificially, by sonic treatment. (c) The toxin within the organism is prevented from reaching the site of action by some unknown mechanism which operates *in vivo*. The first hypothesis seems least probable in view of the finding that toxin solutions are not affected by the sonic treatment. On the other hand the available data do not permit a choice between the other two possibilities. In connexion with hypothesis (c), it may further be suggested that the toxin within the organisms is destroyed or inactivated in the host by the leucocytes. It will be recalled that according to van Ermengem (1897) and other investigators (Roemer, 1900; Landmann, 1904, quoted by Bengtson, 1924) *Clostridium botulinum* organisms, when injected intraperitoneally, undergo rapid phagocytosis. This explanation implies that the power of the white cells to cope with the 'bound' toxin is quite considerable. This interesting phenomenon, the details of which remain obscure, deserves further investigation.

In the system of non-multiplying organisms it was possible to determine certain of the essential factors which govern toxin production. The requirements for amino acids, glucose, inorganic phosphates and magnesium seem to indicate that conditions for *de novo* synthesis of protein must be satisfied. Rigorous proof for this claim might be provided by experiments involving labelled amino acids. However, the arrest of toxin production resulting from the omission of even a single essential amino acid (which the organism is unable to synthesize) strongly supports this assumption. Furthermore, the higher concentrations of amino acids required for optimal toxin production as compared with those necessary for multiplication, furnish additional evidence in favour of this hypothesis.



The requirement for glucose suggests that a rich energy source is also essential for toxin synthesis. Likewise, the dependence on inorganic phosphate points to a need for energy rich compounds (e.g. adenosine triphosphate). The importance of adenosine triphosphate or related compounds for peptide bond synthesis as suggested by Lipmann (1949) was demonstrated by Peterson & Greenberg (1952).

Although no need for magnesium ion could be demonstrated directly, the inhibition of toxin formation by EDTA and its specific reversal by magnesium ion indicates an essential function of this ion in the system described. It has been shown (Snoke, Yanari & Bloch, 1953) that magnesium is needed for enzymic synthesis of glutathione. Whether additional factors (e.g. components of nucleic acids or vitamins) are involved in toxin synthesis cannot be answered at present in view of the difficulties inherent in removal from whole cells of substances not easily dissociable.

*Rate of toxin formation.* Little toxin is formed during the active period of multiplication. Since the toxin is formed intracellularly and since old organisms (30–40 hr.) autolyse rapidly, it follows that most of the toxin is formed within a short period of time, early in the phase of decline. The kinetic studies with resting organisms (see Fig. 4) showed this very clearly. Our findings are in disagreement with observations of Raynaud, Turpin, Mangalo & Bizzini (1954, 1955). These authors, using a different experimental set-up, concluded that the toxins of *Clostridium botulinum* and *Corynebacterium diphtheriae* are formed mainly during the logarithmic phase of growth. On the other hand, our observations are in accord with the findings of Pappenheimer (1955) that toxin production by *C. diphtheriae* starts only after cessation of the rapid-growth phase.

Autolysis results in the liberation of the cell contents into the surrounding medium. It is, however, not known whether the various enzymes liberated in this process are preserved or undergo destruction. Since neither autolysed nor artificially-disrupted organisms are able to synthesize toxin, it remains to be seen whether preservation of certain particulate cell components, or addition of soluble factors, is needed for toxin formation by a cell-free preparation.

Chloramphenicol and chlortetracycline were found to inhibit protein synthesis by resting staphylococci or cell fragments (Gale & Folkes, 1953, 1955). The system studied by us was less sensitive to these agents and was not affected at all by penicillin. However, this difference in behaviour towards antibiotics does not necessarily indicate that there is a qualitative difference in the mechanism of protein synthesis in the different systems.

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**An Assay of Iron Protoporphyrin based on the Reduction of Nitrate by a variant Strain of *Staphylococcus aureus*; Synthesis of Iron Protoporphyrin by Suspensions of *Rhodopseudomonas spheroides***

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**SUMMARY:** A growth requirement for haematin by a streptomycin-resistant strain of *Staphylococcus aureus* was abolished by adding pyruvate or acetate. Growth in the absence of haematin was improved by addition of purines and uracil. Under anaerobic conditions growth occurred in the presence of nitrate when haematin was added. Suspensions of organisms grown in the absence of haematin reduced nitrate to nitrite when incubated in buffered glucose with haematin, and the amount of nitrite formed was proportional to the concentration of haematin over a range from 0.03 to 0.25  $\mu\text{m-mole/ml}$ . This method was used to assay iron protoporphyrin formed by suspensions of *Rhodopseudomonas spheroides*. This organism synthesizes iron protoporphyrin as well as free porphyrins when incubated anaerobically in the light with  $\delta$ -aminolaevulinic acid, iron salts and an oxidizable substrate; cobalt ions inhibit the formation of iron protoporphyrin.

Few bacteria have been found to require haematin for growth though it is a nutrient essential for many protozoa (Lwoff, 1951). The best known example of a requirement for haematin in bacteria is that of *Haemophilus influenzae*; growth is also promoted by the corresponding free porphyrin, protoporphyrin (Granick & Gilder, 1946; Gilder & Granick, 1947). Jensen & Thofern (1953*a*) isolated a streptomycin-resistant strain of *Staphylococcus aureus* which required added haematin for growth under certain conditions. This organism differs from *Haemophilus influenzae* in that haematin is not replaced by other iron porphyrins or by protoporphyrin. This staphylococcus variant when grown with haematin contains a cytochrome system similar to that of the parent strain grown in the absence of haematin (Jensen & Thofern, 1953*a*, 1954). The variant organism grows in the absence of haematin in a liquid medium containing tryptic digest of casein and glucose, and under these conditions the cells contain no detectable cytochrome. Also, they neither consume oxygen with glucose as substrate nor possess catalase activity (Jensen & Thofern, 1953*a*). Addition of haematin to suspensions of the exacting variant restores both respiratory and catalase activity (Jensen & Thofern, 1953*b*).

In the present work the variant strain of Jensen & Thofern was first examined with a view to obtaining a method for the microbiological assay of haematin or other forms of iron protoporphyrin. Since the conditions under which it needs this substance for growth are limited, an assay method which used growth response as criterion was not practicable. However, the ability of

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suspensions of the variant staphylococcus to reduce nitrate to nitrite was found to depend on the concentration of haematin added. This assay method has been used to study synthesis of iron protoporphyrin by suspensions of *Rhodopseudomonas spheroides*, a member of the Athiorhodaceae (van Niel, 1944). The haematin-like material formed by *Rhodopseudomonas spheroides* is hereafter referred to as iron protoporphyrin since it is not known whether the iron is in the ferric or ferrous state.

The photosynthetic bacteria are rich in haematin compounds as well as bacteriochlorophyll (Elsden, 1954; Vernon & Kamen, 1954; Kamen, 1955). Some of them also form considerable amounts of free porphyrin under conditions where synthesis of bacteriochlorophyll is limited by iron deficiency (Lascelles, 1955). The concurrent formation of free iron protoporphyrin and porphyrins from  $\delta$ -aminolaevulinic acid has been studied in the present work.

#### METHODS

**Organisms.** *Staphylococcus aureus*, the streptomycin-resistant variant requiring haematin for growth (Strain SG 511 Var) was obtained from Dr J. Jensen. Some of its properties have been described by Jensen & Thofern (1953*a-c*, 1954). It was maintained by monthly subculture on chocolate agar slopes which were incubated 18 hr. at 37° and then stored at 4°. Details about *Rhodopseudomonas spheroides* have been given previously (Lascelles, 1956).

**Media.** The medium (medium A) used for the preparation of suspensions of the staphylococci contained (% w/v): peptone (Evans Medical Supplies Ltd., Liverpool, England), 1.4; Marmite (a commercial autolysate of yeast), 0.4; glucose, 1; K<sub>2</sub>HPO<sub>4</sub>, 0.8; sodium acetate (trihydrate), 0.16; the pH value was 7.4. It was essential to prepare this medium immediately before use to obtain organisms with maximum activity. The basal medium (medium B) for growth tests was based on acid hydrolysed casein (vitamin-free) and was similar to medium B of Lascelles & Woods (1952) except that the glucose concentration was increased to 0.05 M; the glucose was added after the medium had been autoclaved.

*Rhodopseudomonas spheroides* was grown on the chemically defined medium S described previously (Lascelles, 1956).

**Growth experiments with the staphylococcus.** Growth tests were carried out in 150 × 18 mm. tubes containing 2 ml. (final volume) of medium B, which was autoclaved at 10 lb./sq.in. for 7 min.; glucose, haematin and protoporphyrin were added after autoclaving. The inoculum was an aqueous suspension of organisms from an 18 hr. culture on a chocolate agar slope; each tube was sown with about  $4 \times 10^5$  organisms (0.12  $\mu$ g. dry wt.) and incubated at 37° in a sloped position in air or in an atmosphere of 5% (v/v) CO<sub>2</sub> in H<sub>2</sub> in a McIntosh and Fildes jar.

Growth was measured on an EEL photoelectric colorimeter (Evans Electro-selenium Co. Ltd., Harlow, Essex, England) using 6 mm. tubes and a neutral density filter; the instrument was adjusted to give a reading of zero with uninoculated medium. A culture containing 0.14 mg. dry wt. organisms/ml.



gave a reading of 10; there was a linear relationship between instrument reading and dry weight of organisms up to a scale reading of 40.

*Experiments with suspensions of the variant staphylococcus.* Erlenmeyer flasks (250 ml.) containing 125 ml. medium A were sown with 0.25 ml. of an aqueous suspension containing about  $4 \times 10^8$  organisms/ml. derived from an 18 hr. chocolate agar slope; they were incubated for 18 hr. at  $37^\circ$ . The organisms were harvested by centrifuging, washed in 100 ml. 0.85% (w/v) NaCl and suspended in water to give a concentration of 7–9 mg. dry wt. organisms/ml. The reaction mixture used for the assay of iron protoporphyrin was as follows: organisms, 2–3 mg. dry wt.; phosphate buffer pH 6.9, 0.1 M;  $\text{NaNO}_3$ , 0.008 M; glucose, 0.02 M; after addition of haematin or the samples under assay the final volume was 2.5 ml. Incubation was in  $120 \times 15$  mm. tubes for 3–5 hr. at  $37^\circ$ .

*Experiments with Rhodopseudomonas spheroides.* Organisms were grown anaerobically in the light and harvested by the methods described previously (Lascelles, 1956). Synthesis of porphyrins and iron protoporphyrin was followed in mixtures containing: washed organisms, 2.5–4 mg. dry wt.; phosphate buffer pH 6.9, 0.08 M;  $\text{MgSO}_4$ ,  $8 \times 10^{-4}$  M; sodium fumarate, 0.004 M;  $\delta$ -aminolaevulinic acid hydrochloride, 0.002 M; iron citrate, various concentrations (see below); the final volume was 2.5 ml. Incubation was at  $34^\circ$  in Thunberg tubes filled with  $\text{H}_2$ ; the tubes were stood in front of banks of tungsten lamps (40–60 W.) and the intensity of light falling on them was about 250 ft.c.

*Estimation of nitrite.* The concentration of nitrite formed by suspensions of the staphylococcus was estimated in the supernatant fluid after removal of the organisms by centrifuging. It was estimated colorimetrically by a method based on the Griess-Ilosvay reaction, similar to that described by Rider & Mellon (1946). The reagents were: (a) sulphanilic acid, 0.3% (w/v) dissolved in a mixture of N-HCl and 8.5 N-acetic acid; (b)  $\alpha$ -naphthylamine, 0.6% (w/v) in 0.2 N-HCl. The sample (0.1–1 ml.) was mixed with 0.5 ml. sulphanilic acid reagent; after standing for 3 min., 0.25 ml.  $\alpha$ -naphthylamine and 0.5 ml. 2 M-sodium acetate were added and the final volume brought to 5 ml. with water. The colour developed after 20–30 min. was read on the EEL photoelectric colorimeter (Evans Electroselenium Co. Ltd.,) using 11 mm. tubes and EEL filter no. 623 (transmission peak at  $520 \text{ m}\mu$ ). Sodium nitrite was used as standard; stock solutions (0.1 M) were stored in the dark at  $0^\circ$  and were diluted immediately before use. The standard series, included in each assay, ranged from 0.2 to  $2 \mu\text{m}$ -moles  $\text{NaNO}_2$ /ml.; the relation between EEL reading and concentration of nitrite was linear between these limits.

*Chemical estimation of haematin.* This was estimated after conversion to the pyridine haemochromogen derivative. Samples containing 5–50  $\mu\text{m}$ -moles haematin in 0.01 N-NaOH were mixed with pyridine (50%, v/v) and reduced with about 5 mg. sodium dithionite. The optical density at  $558 \text{ m}\mu$ . was measured on a spectrophotometer (Model SP-600, Unicam Instruments Ltd., Cambridge, England) and the concentration of haematin calculated from the extinction coefficient for the pyridine haemochromogen derivative of protohaem determined by Drabkin (1942).



*Estimation of porphyrins.* The methods described by Lascelles (1956) for the determination of total porphyrin and the estimation of the relative proportion of copro- and protoporphyrin were used.

*Special chemicals.* Solutions of haematin were prepared by dissolving 2–3 mg. haemin (ferric protoporphyrin chloride obtained from British Drug Houses Ltd., Dorset, England) in 5 ml. 0.01 N-NaOH, thereby converting it to haematin (Lemberg & Legge, 1949); dilutions were made in water. The concentration of haematin in each stock solution was determined spectrophotometrically. Fresh solutions were used since their biological activity decreased rapidly even on storage in the dark at 0°.

Protoporphyrin was obtained from Light and Co. Ltd. (Colnbrook, Bucks, England). Stock solutions (in 0.01 N-NaOH) contained about 500  $\mu$ g. protoporphyrin/ml.; the concentration was determined spectrophotometrically (Grinstein & Wintrobe, 1948).

Stock solutions of iron citrate ( $4 \times 10^{-3}$  M with respect to iron) were made by dissolving 157 mg. ferrous ammonium sulphate (hexahydrate) and 236 mg. sodium citrate (dihydrate) in 100 ml. water.

## RESULTS

### *Requirement of growing cultures of the variant staphylococcus for haematin*

*Aerobic growth.* Haematin was required for growth on medium B when incubation was in air; maximum growth was attained with  $3 \times 10^{-8}$  M haematin (Fig. 1). Protoporphyrin (up to  $10^{-5}$  M) was inactive even when the medium was supplemented with iron ( $2 \times 10^{-5}$  M). These observations are in accord with those of Jensen & Thofern (1953a).

*Replacement of haematin.* Growth of the staphylococci occurred in the absence of added haematin when medium B was supplemented with sodium acetate or pyruvate. Under these conditions the further addition of a mixture containing adenine, xanthine and uracil brought the amount of growth up to that attained by adding haematin alone (Table 1). The nucleic acid derivatives were ineffective in the absence of acetate or pyruvate.

The concentration at which pyruvate and acetate were active was relatively low, suggesting that they were not acting merely as sources of energy (Table 2). Moreover, glucose was required for growth with acetate, while in the presence of pyruvate only slight growth occurred when glucose was omitted (Table 2). Glucose apparently provided the major source of energy for growth while pyruvate and acetate behaved as essential metabolites possibly needed for biosynthetic processes. They were not acting as precursors of haematin for the staphylococcus since organisms grown in their presence contained no detectable cytochrome nor did they show oxygen uptake when incubated with glucose. On the other hand, organisms grown with haematin contained cytochrome pigments and showed vigorous oxygen uptake with glucose as substrate.

*Anaerobic growth in the presence of nitrate.* The variant staphylococcus did not grow on medium B under anaerobic conditions either in the presence or absence of haematin. When nitrate was added to the medium growth occurred,

Table 1. *Growth response of the staphylococcus variant to haematin, acetate, pyruvate or nucleic acid derivatives*

Medium B was supplemented as shown. The mixture of nucleic acid derivatives (PU mixture) contained adenine, xanthine and uracil (each  $10^{-4}$  M final concn.). Incubation was for 24 hr. in air.

Additions				Relative degree of growth (EEL reading)
Haematin ( $10^{-6}$ M)	PU mixture	Na-acetate ( $10^{-2}$ M)	Na-pyruvate ( $10^{-2}$ M)	
—	—	—	—	0
+	—	—	—	33
—	+	—	—	0
—	—	+	—	16
—	+	+	—	27
—	—	—	+	29
—	+	—	+	35
+	+	—	—	33
+	+	+	—	34
+	+	—	+	35

Table 2. *Growth response of the haematin-requiring staphylococcus variant to pyruvate, acetate and glucose*

Medium B without glucose was supplemented with adenine, xanthine and uracil (each  $10^{-4}$  M) with further additions as shown. Incubation was for 24 hr. in air.

Additions			Relative degree of growth (EEL reading)
Na-acetate (M)	Na-pyruvate (M)	Glucose (0.05 M)	
—	—	+	0
$3 \times 10^{-4}$	—	+	5
$1 \times 10^{-3}$	—	+	15
$3 \times 10^{-3}$	—	+	28
$1 \times 10^{-2}$	—	+	29
$1 \times 10^{-2}$	—	—	0
—	$3 \times 10^{-4}$	+	10
—	$1 \times 10^{-3}$	+	18
—	$3 \times 10^{-3}$	+	29
—	$1 \times 10^{-2}$	+	34
—	$1 \times 10^{-2}$	—	9

but only in the presence of haematin; nitrite was found in such cultures. The concentration of haematin required for maximum growth on nitrate was similar to that needed for aerobic growth in the absence of nitrate; maximum growth under both conditions was also similar (Fig. 1). This suggests that haematin (probably in the form of a haemoprotein) is a component of the nitratase enzyme system of the organism and that linked reactions between hydrogen donors and nitrate can provide energy necessary for growth.

*Reduction of nitrate by suspensions of the variant staphylococcus and the assay of haematin*

*Requirements for nitrate reduction.* The organism grew well on the complex medium A which contained acetate but not haematin; the yield of organisms after incubation for 18 hr. was about 0.5 mg. dry wt./ml. culture. Organisms

harvested from this medium formed only traces of nitrite from nitrate when incubated with buffered glucose, but active reduction occurred when haematin was added (Table 3). Protoporphyrin (up to  $10^{-5}$  M) did not replace haematin even in the presence of added iron ( $10^{-5}$  M). The organisms reduced nitrate to some extent in the presence of phosphate and haematin only; presumably endogenous substances in the cells served as hydrogen donors. Addition of glucose greatly increased the formation of nitrite (Table 3).

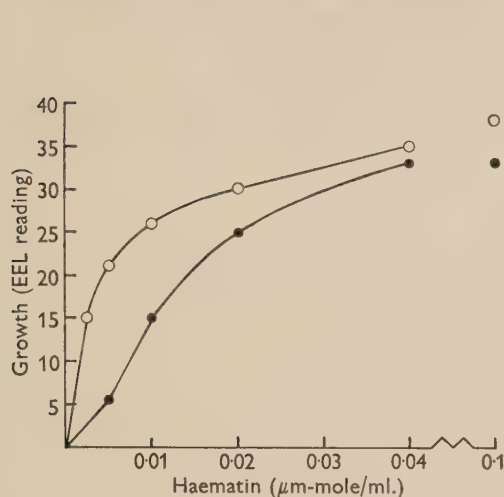


Fig. 1

Fig. 1. Effect of haematin on growth of the staphylococcus variant. The cultures were incubated for 22 hr. in medium B in air (●—●) or in medium B with 0.01 M-NaNO<sub>3</sub> under an atmosphere of 5% CO<sub>2</sub> (v/v) in H<sub>2</sub> (○—○).

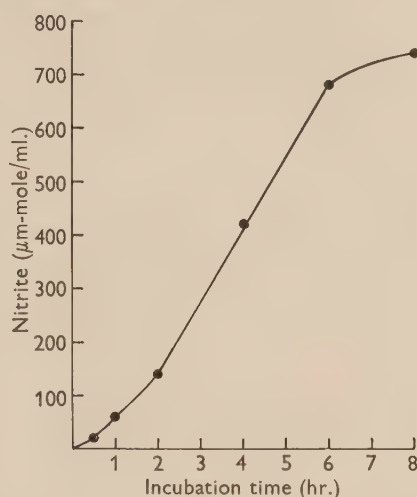


Fig. 2

Fig. 2. Rate of nitrite formation from nitrate by suspensions of the staphylococcus variant. The organisms (1.3 mg./ml. dry wt.) were incubated in 0.1 M-phosphate buffer, pH 6.9, with 0.02 M-glucose, 0.008 M-NaNO<sub>3</sub> and  $6 \times 10^{-6}$  M-haematin.

Table 3. Requirements for reduction of nitrate to nitrite by suspensions of the staphylococcus variant

Organisms (3.2 mg. dry wt.) were incubated for 5 hr. in 0.1 M-phosphate buffer (pH 6.9) with the additions shown; the final volume was 2.5 ml.

Additions			Nitrite formed (μm-mole/ml.)
NaNO <sub>3</sub> (0.008 M)	Glucose (0.02 M)	Haematin (10 <sup>-6</sup> M)	
+	+	+	650
—	+	+	0
+	—	+	125
+	+	—	1

*Course of the reaction.* The rate of nitrite formation increased gradually during the first 2 hr. of incubation, but became linear thereafter (Fig. 2). The initial lag may have been due to the formation of an adaptive enzyme; the formation of nitratase by suspensions of coliform organisms is an adaptive process (Pollock, 1946). Inclusion of nitrate in the growth medium did not alter the behaviour of the harvested suspensions.

The amount of nitrite formed was only a small fraction (*c.* 9%) of the theoretical value for complete reduction of the added nitrate. This low yield was not due to further reduction of nitrite (for instance to ammonia) since added nitrite was recovered quantitatively after incubation with organisms in the presence of buffered glucose and haematin.

*The assay of haematin.* The amount of nitrite formed from nitrate was proportional to the amount of haematin over a range of concentration from 0.04 to 0.25  $\mu\text{m-mole/ml.}$  (Fig. 3). In assays of haematin, organisms were suspended in buffered glucose with nitrate and incubated for 4–5 hr. with

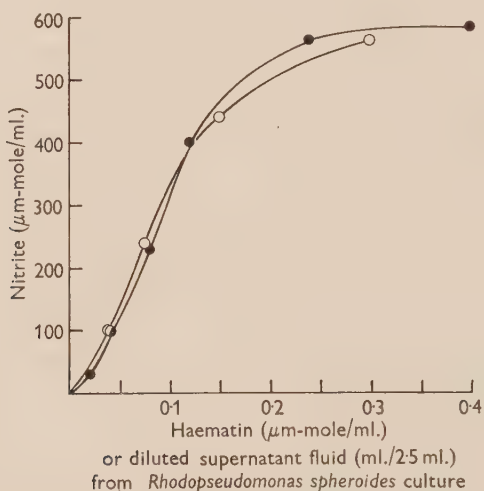


Fig. 3. The effect of concentration of haematin (●—●) or of supernatant fluid from *Rhodopseudomonas spheroides* (○—○) on the formation of nitrite by suspensions of the staphylococcus variant. The organisms (1.2 mg./ml. dry wt.) were incubated for 5 hr. in a mixture of 0.1 M-phosphate buffer, pH 6.9, 0.008 M- $\text{NaNO}_3$ , and 0.02 M-glucose, with addition of haematin or diluted sample.

addition of the sample under assay. A standard series was included in each assay with concentrations of haematin ranging from 0.03 to 0.4  $\mu\text{m-mole/ml.}$  and a curve was constructed relating the concentration of nitrite formed to the amount of haematin. The concentration of haematin in the unknowns was calculated from the standard curve. Haematin could be estimated by this method with an accuracy of  $\pm 20\%$ ; the assay was about 100 times more sensitive than the spectrophotometric method for the estimation of pyridine haemochromogen (Drakbin, 1942), which is suitable only for concentrations of 5  $\mu\text{m-mole/ml.}$  or more.

#### *The formation of iron protoporphyrin by suspensions of Rhodopseudomonas spheroides*

Suspensions of *Rhodopseudomonas spheroides* form porphyrins (mainly coproporphyrin type III) when incubated anaerobically in the light with glycine and  $\alpha$ -oxoglutarate (Lascelles, 1956).  $\delta$ -Aminolaevulinic acid (ALA) is also con-



verted to porphyrins under similar conditions and behaves as if it were an intermediate between glycine +  $\alpha$ -oxoglutarate and the porphyrins. Coproporphyrin type III is formed predominantly from ALA when the organisms are incubated in phosphate buffer with  $Mg^{++}$  only; the further addition of an oxidizable substrate, such as fumarate, together with iron salts, results in the formation of both protoporphyrin and coproporphyrin.

Table 4. *The requirements of Rhodopseudomonas spheroides for synthesis of iron protoporphyrin and porphyrin from  $\delta$ -aminolaevulinic acid*

Organisms (1.2 mg. dry wt./ml. in both experiments) were incubated in 0.08 M-phosphate (pH 6.9) containing  $8 \times 10^{-4}$  M- $MgSO_4$  and additions shown; total volume, 2.5 ml. Incubation was in the light under  $H_2$  for 22 hr. (Expt. 1) and 15 hr. (Expt. 2).

Expt.	Additions			Iron protoporphyrin ( $\mu$ m-mole/ml.)	Porphyrin* ( $\mu$ m-mole/ml.)	Percentage of ether-soluble porphyrin present as†	
	<i>d</i> -amino- laevulinic acid (M)	Iron citrate ( $2 \times 10^{-5}$ M)	Carboxylic acid ( $4 \times 10^{-3}$ M)			Copro- porphyrin	Proto- porphyrin
1	$2 \times 10^{-3}$	—	—	< 0.5	101	100	0
		+	—	< 0.5	99	100	0
		—	Fumarate	0.6	72	92	8
		+	Fumarate	30	65	80	20
		+	Lactate	32	83	78	22
		+	Acetate	34	64	81	19
	0	+	Fumarate	< 0.5	0	.	.
2	$5 \times 10^{-4}$	+	Fumarate	0.7	17	.	.
	$1 \times 10^{-3}$	+	Fumarate	6	34	78	22
	$2 \times 10^{-3}$	+	Fumarate	18	77	76	24
	$4 \times 10^{-3}$	+	Fumarate	23	103	84	16
	$2 \times 10^{-3}$	—	Fumarate	< 0.5	94	95	5
	$2 \times 10^{-3}$	+	—	< 0.5	99	98	2

\* Total free porphyrin.

† The ether-soluble porphyrins comprise 96 % of the total porphyrin.

Further experiments showed that the suspensions also formed a substance which promoted growth of the staphylococcus in the absence of haematin; it was produced only under conditions which favoured the formation of protoporphyrin. The active substance was identified as iron protoporphyrin by several methods (see later) and has been assayed by the nitrataase method. It was present in the supernatant fluid after removal of the organisms by centrifugation; no attempt was made to determine the iron protoporphyrin which may have been present either in free or bound form within the organisms.

*Conditions for the formation of iron protoporphyrin.* Iron protoporphyrin as well as protoporphyrin and coproporphyrin were formed by *Rhodopseudomonas spheroides* when incubated anaerobically in the light with ALA, fumarate (or other oxidizable substrates) and iron (Table 4). These compounds were all essential for the synthesis of maximum amounts of iron protoporphyrin and of protoporphyrin; formation of coproporphyrin, however, occurred in the absence of fumarate and iron. The concentration of iron required for maximum

production of iron protoporphyrin was about five times greater than that needed for the formation of protoporphyrin (Table 5).

Table 5. *The effect of concentration of iron on production of iron protoporphyrin and protoporphyrin by Rhodopseudomonas spheroides*

Organisms (1.2 mg. dry wt./ml.) were suspended in 0.08 M-phosphate, pH 6.9,  $8 \times 10^{-4}$  M-MgSO<sub>4</sub>,  $4 \times 10^{-3}$  M-fumarate and  $2 \times 10^{-3}$  M-ALA with addition of iron (as the citrate). Incubation was for 22 hr. in the light under H<sub>2</sub>.

Added iron (M)	Iron proto- porphyrin ( $\mu$ m-mole/ml.)	Porphyrin* ( $\mu$ m-mole/ml.)	Percentage ether-soluble porphyrin present as †	
			Coproporphyrin	Protoporphyrin
0	< 0.5	98	97	3
$4 \times 10^{-6}$	2	92	89	11
$1 \times 10^{-5}$	11	85	76	24
$2 \times 10^{-5}$	24	85	85	15
$4 \times 10^{-5}$	30	81	85	15
$1 \times 10^{-4}$	32	85	89	11

\* Total free porphyrin.

† The ether-soluble porphyrins comprise 96 % of the total porphyrin.

Iron protoporphyrin was not formed when the organisms were incubated in the light with protoporphyrin up to  $50 \mu$ m-mole/ml. in the presence of fumarate and iron. Therefore, the iron protoporphyrin found in systems containing ALA presumably did not arise by chemical or enzymic reaction between iron salts and the protoporphyrin formed concurrently from ALA.

Neither porphyrins nor iron protoporphyrin were formed when the organisms were incubated anaerobically in the dark. Little or no iron protoporphyrin was synthesized by aerobically-grown organisms incubated aerobically in the dark with ALA, fumarate and iron; under these conditions protoporphyrin as well as coproporphyrin is formed though the yield of porphyrin is only about 20–30 % of that formed during anaerobic incubation in the light (Lascelles, 1956).

*Effect of cobalt ions.* The synthesis of iron protoporphyrin from ALA was inhibited by Co<sup>++</sup> at concentrations similar to that of the iron present (Table 6). Synthesis was not affected by Zn<sup>++</sup>, Pb<sup>++</sup>, Ni<sup>++</sup> nor by ammonium molybdate (each tested at  $10^{-5}$  M).

Cobalt also inhibited the formation of protoporphyrin (Table 6), suggesting that its locus of action may be at a stage before the insertion of iron into the porphyrin nucleus. Cobalt has been found to inhibit incorporation of radioactive glycine into haem by preparations of rabbit bone marrow (Laforet & Thomas, 1956).

*Identification of iron protoporphyrin.* The response to haematin of the staphylococcus in the nitrataase assay was similar to that obtained with graded amounts of supernatant fluid obtained from experiments with *Rhodopseudomonas spheroides* (Fig. 3). Also, a strictly additive effect was obtained on addition of supernatant fluid together with suboptimal concentrations of haematin.

Further evidence that the material formed by *Rhodopseudomonas spheroides* was iron protoporphyrin was obtained by examination of concentrates by paper chromatography and by spectroscopy. Pooled supernatant fluids from typical experiments were adjusted to pH 4 with acetic acid and extracted with ether; the ethereal extracts contained both porphyrins and iron protoporphyrin. The free porphyrins were removed by extraction with 1.4N-HCl, leaving the iron protoporphyrin in the ether phase. After washing with water,

Table 6. *Effect of cobalt ions on formation of iron protoporphyrin and porphyrins by Rhodopseudomonas spheroides*

Organisms (1.4 mg. dry wt./ml.) were suspended as in Table 5; iron was added as the citrate. Incubation was for 21 hr. in the light under  $H_2$ .

Additions		Iron proto- porphyrin ( $\mu$ m-mole/ml.)	Porphyrin* ( $\mu$ m-mole/ml.)	Percentage ether-soluble prophyrin present as†	
Iron (M)	CoCl <sub>2</sub> (M)			Copro- porphyrin	Proto- porphyrin
0	0	0.2	104	98	2
$1 \times 10^{-5}$	0	8	96	82	18
$4 \times 10^{-5}$	0	32	93	84	16
$1 \times 10^{-5}$	$4 \times 10^{-6}$	7	93	95	5
$4 \times 10^{-5}$	$4 \times 10^{-6}$	10	86	94	6
$1 \times 10^{-5}$	$1 \times 10^{-5}$	2	89	100	0
$4 \times 10^{-5}$	$1 \times 10^{-5}$	5	93	100	0
$1 \times 10^{-5}$	$4 \times 10^{-5}$	<0.05	98	100	0
$4 \times 10^{-5}$	$4 \times 10^{-5}$	<0.05	96	100	0

\* Total free porphyrin.

† The ether-soluble porphyrins comprised 96 % of the total porphyrin.

the ether was distilled off and a brown residue with high activity for the variant staphylococcus remained. This material gave a single spot (visible under ultraviolet irradiation) with an  $R_F$  value identical with that of haematin, when examined by the method of Chu & Chu (1955) using reverse-phase paper chromatography. Solutions of the concentrate in 0.01N-NaOH were converted to the pyridine haemochromogen derivative and examined with a Hartridge reversion spectroscope (R. and J. Beck Ltd., London, W. 1). The absorption bands at 557.5 and 528.7 m $\mu$ . coincided with those found with pure haematin extracted and treated under the same conditions.

## DISCUSSION

The ability of acetate and pyruvate to replace haematin for growth of the variant staphylococcus suggests that they are products, though not necessarily direct ones, of a reaction in which haematin is concerned. Organisms grown with haematin contain cytochromes (Jensen & Thofern, 1953*a*, 1954) and these presumably participate in the ultimate stages of electron transfer to oxygen. When the cytochrome system is available to the organism, pyruvate and acetate may be formed from precursors derived by oxidation of glucose or of amino acids. In the absence of haematin pyruvate and acetate, if formed, do

not accumulate in amounts sufficient to promote growth of the organism. Acetate is formed by the anaerobic dissimilation of pyruvate by other strains of *Staphylococcus aureus* (Krebs, 1937). It is likely that the variant can convert pyruvate to acetate irrespective of the presence of haematin and that the metabolic block caused by the absence of haematin results in a complete or partial inability to form pyruvate. An examination of the metabolism of glucose and other carbon compounds by the organism grown with and without haematin is clearly necessary before the growth-promoting action of pyruvate and acetate can be fully understood.

Both pyruvate and acetate are known to be essential metabolites for other micro-organisms. Pyruvate is needed for growth of a saprophytic treponeme (S-69) on a chemically defined medium (Steinman, Oyama & Schulze, 1954). Acetate is essential for growth of some lactobacilli in the absence of lipoic (thioctic) acid; this cofactor is needed for the oxidative decarboxylation of pyruvate to acetate (Gunsalus, 1954). Lipoic acid had no effect on the requirement of the staphylococcus variant for pyruvate or acetate.

The stimulatory effect of nucleic acid derivatives on growth with pyruvate or acetate in the absence of haematin suggests that precursors of these compounds are formed most efficiently when the cytochrome system is available. This is further suggested by the observation that uracil is essential for the anaerobic growth of *Staphylococcus aureus* but is not necessary for aerobic growth (Richardson, 1936).

Nitrate is utilized by the staphylococcus variant as a terminal hydrogen acceptor in growing cultures just as effectively as oxygen, provided that haematin is present. The organism is therefore capable of true dissimilatory nitrate reduction as defined by Kluver (1953) and Verhoeven & Goos (1954). The experiments with growing cultures and with suspensions of the staphylococcus variant indicate that haematin, probably as a haemoprotein, participates in the reduction of nitrate by this organism. The nitrate reductase may therefore be similar to that in *Haemophilus influenzae*; in this organism nitrite production from nitrate occurs in cultures grown with haematin (or with protoporphyrin) but is not found in cultures grown with other iron porphyrins which support growth (Granick & Gilder, 1946; Smith, Hale & O'Callaghan, 1953). There is also evidence that cytochromes participate in the reduction of nitrate by cell-free extracts of *Escherichia coli* (Sato & Egami, 1949; Sato & Niwa, 1952). A similar function of cytochromes in the reduction of sulphate by *Desulphovibrio desulphuricans* is suggested by the work of Postgate (1955, 1956). Haematin is not, however, a component of nitrate reductase prepared in purified form from *E. coli* and *Neurospora*; in these preparations reduction of nitrate to nitrite is catalysed by a molybdo-flavoprotein (Nicholas & Nason, 1954, 1955). It is possible, therefore, that there may be several types of nitrate reductase enzymes in micro-organisms.

The synthesis of iron protoporphyrin by *Rhodopseudomonas spheroides* from  $\delta$ -aminolaevulinic acid occurs simultaneously with the formation of protoporphyrin. The present experiments provide no indication of the stage at which iron is inserted into the porphyrin nucleus. If it is incorporated after



the formation of free protoporphyrin it might be expected that the organisms would form iron protoporphyrin when incubated with protoporphyrin and iron, but this was not found. Protoporphyrin may not penetrate into the cells; on the other hand, iron may be attached to a precursor of protoporphyrin before completion of the final product. Iron is also necessary for the synthesis of free protoporphyrin from ALA but is required at a lower concentration (*c.* fivefold) than is needed for demonstrable formation of iron protoporphyrin. The experiments do not show whether the function of iron in the conversion of ALA to protoporphyrin is connected with the synthesis of iron protoporphyrin.

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## Nutritional Studies on *Vibrio cholerae*

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**SUMMARY:** One hundred and fifty-eight strains of vibrio, predominantly *Vibrio cholerae*, were tested for their nutritional requirements. Half of the strains were able to grow on a simple inorganic medium with ammonium ions as the sole source of nitrogen; the other strains required purines in addition. The simplest purine which produced adequate growth was hypoxanthine. The purine-requiring strains were able to grow on inorganic media containing human, rabbit or goat serum, but not on the serum from mouse, rat, guinea pig or horse.

Little information is available about the nutritional requirements of *Vibrio cholerae*. Some strains are non-exacting, as they are capable of growth in minimal media containing only ammonium salts and glucose as nitrogen, carbon and energy sources (Anderson, 1946; Saxena, Bhaskaran, Agarwala & Shrivastava, 1953); other strains do not grow in such simple media, indicating that they need additional factors for growth. These factors are obviously available in ordinary protein digest media in which all strains grow profusely. This investigation is concerned with elucidating the precise constituents of these complex media which are essential for the growth of *V. cholerae*.

### METHODS

*Organisms tested.* *Vibrio* strains were collected for this investigation from various laboratories in India and from the National Collection of Type Cultures, Colindale, London N.W. 9, England. One hundred and fifty-eight strains were true *Vibrio cholerae* O Group I (Gardner & Venkatraman, 1935) including the specific antigenic types Inaba, Ogawa and Rough forms. Ten strains were non-agglutinable by the specific cholera anti-sera and were not cholera vibrios, presumably isolated from water sources in India.

*Auxanographic technique.* For the differentiation between exacting and non-exacting strains, thoroughly washed suspensions of each strain from overnight growth on nutrient agar, were streaked on a solid minimal medium. The medium contained ammonium sulphate (0.1 %), glucose (0.1 %),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.02 %), NaCl (0.5 %) and  $\text{K}_2\text{HPO}_4$  (0.1 %) in distilled water; the pH value was adjusted to 8.0, and the medium solidified with 2 % Bacto Agar (Saxena *et al.* 1953). Colonies of non-exacting strains appeared after overnight incubation at 37°; no growth could be seen with the exacting strains.

The nutritional requirements of the exacting strains were examined by the technique of Pontecorvo (1949). Possible growth-promoting materials such as

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Difco Casamino acids, yeast nucleic acid, *p*-amino benzoic acid, pantothenic acid, glutathione, biotin, vitamin B<sub>12</sub>, nicotinamide, and thiamine, were tested by adding them dropwise, individually or in groups, on to the surface of the basal medium seeded with a washed suspension of the test strain, to see whether growth of the organism could be obtained. Test substances were made up to a concentration of 1 mg./ml. with the exception of biotin and vitamin B<sub>12</sub> which were employed in strengths of 1 and 50 µg./ml., respectively.

## RESULTS

*Exacting and non-exacting strains.* Strains were equally distributed between the two groups, irrespective of their antigenic character (Table 1). Study of the 79 exacting strains, by the auxanographic technique, showed that with the exception of one strain (Ogawa M74) which required methionine for growth, all grew in the presence of yeast nucleic acid.

Table 1. *Distribution of vibrios in two nutritional groups*

Nutrition	strains of vibrios				Total
	Inaba	Ogawa	Rough no. of strains	N.A.G.	
Non-exacting	39	36	2	2	79
Exacting	38	31	2	8	79

N.A.G., not agglutinated by specific cholera anti-sera.

Simpler components of the complex polynucleotides of yeast nucleic acid were examined for growth stimulation. In preliminary tests, it appeared possible that the purine or pyrimidine bases themselves might provide the necessary factors for growth of these strains, as complete hydrolysis of yeast nucleic acid by 6N-hydrochloric acid did not abolish the growth-promoting property. Tests carried out with known purines and pyrimidines confirmed this supposition and showed that the growth of exacting strains of *Vibrio cholerae* responded to the addition of purines either in the form of bases (hypoxanthine, adenine or guanine), nucleosides (adenosine or guanosine) or nucleotides (adenylic acid or guanylic acid). The pyrimidines cytosine or uracil were without effect. The most marked growth stimulation was by guanosine in the strains tested (Table 2). One of the supposed precursors of purines is 5-imidazol carboxamide (Greenberg, 1953) but there was no growth stimulation by this compound in the case of *V. cholerae*.

In spite of this general uniformity as regards growth promotion by some component of yeast nucleic acid, certain strain to strain differences were evident. While most of the strains grew well in minimal media supplemented with 0.5% (w/v) yeast nucleic acid certain strains, belonging to the antigenic type Inaba, grew poorly by comparison. It seemed possible that these poorly growing Inaba strains might have a secondary or partial requirement for some other growth factor. The addition of methionine or cystine to the



Table 2. *Growth stimulation of Vibrio cholerae strains by purines*

The strains tested showed no significant growth on the minimal medium which contained only ammonia and glucose (as nitrogen, carbon and energy sources) mineral salts and agar.

Strain	Compounds added to minimal medium									
	Purines					Pyrimidines				
	Hypo-xanthine	Xanthine	Inosine	Adenine	Growth responses		Adenosine	Guanine	Guanosine	Uracil
Inaba 74	++	+	+	+	+	+	+	+	+	—
Inaba 243	++	+	+	+	+	+	+	+	+	—
Inaba 117/53	++	+	+	+	+	+	+	+	+	—
Inaba 113/53	++	+	+	+	+	+	+	+	+	—
Ogawa 842	++	+	+	+	+	+	+	+	+	—
Ogawa 871	+	+	+	+	+	+	+	+	+	—
Ogawa 887	++	+	+	+	+	+	+	+	+	—
Ogawa 51439	+	+	+	+	+	+	+	+	+	—
Rough 243	++	+	+	+	+	+	+	+	+	—
Rough 49515	++	+	+	+	+	+	+	+	+	—

—, no growth; +, growth stimulation; ++, growth stimulation (marked).

medium enabled these strains to grow as well as the others. Such strains, which had a partial requirement for growth factors other than constituents of yeast nucleic acid, formed a third of the collection of exacting Inaba strains of *Vibrio cholerae*. One such strain had a partial requirement for glutathione.

*Tests with animal sera.* The purine dependence of exacting strains of *Vibrio cholerae* was also reflected in experiments carried out with various animal sera. While investigating whether these strains would grow in minimal medium containing serum instead of purines it was found that growth depended on the species of animal from which the serum was obtained (Table 3). These experiments were carried out on solid minimal medium to which various sera were added to a concentration of 25 % (v/v). The sera were heated for 30 min. at 55° before their incorporation in the medium. Saline-washed suspensions of purine-dependent as well as non-exacting strains of *Vibrio cholerae* were inoculated in these media and incubated for 24 hr. It will be seen from Table 3 that sera of man, rabbit and goat were effective in supporting growth of exacting strains while the sera of rat, mouse, guinea pig and horse were not. Identical results were obtained in repeated tests with different batches of sera, which indicated that the differences were between animal species and not between different animals of the same species.

Non-exacting strains of *Vibrio cholerae* were able to grow in all the different serum-containing media, so that there was no evidence of inhibitory activity by any species of serum. Furthermore, with those serum media which did not support the growth of purine-requiring strains, a drop of 0.1 % (w/v) hypoxanthine or yeast nucleic acid solution on the plate stimulated the growth of the organisms around it. It seemed probable therefore that these sera could not support the growth of these strains for the simple reason that they were deficient in purines. As the purine content of the various sera was not known, or determined in these tests, this possibility could only be checked by examining similar purine-requiring strains of other bacterial species, to see whether their growth patterns with these sera were similar.

Table 3 also shows the results of experiments in these serum media with adenine-requiring and non-exacting strains of *Salmonella typhimurium*. The adenine dependent strains were made available by the courtesy of Dr M. Demerec, while the non-exacting strains were derived from them by Dr G. Furness, of this Institute, by phage-mediated transduction to prototrophy. It will be seen that the growth patterns of these strains in the serum-media were identical with the corresponding strains of *Vibrio cholerae*. Essentially similar observations were recorded by Wilson (1945) with strains of group A *Streptococcus pyogenes*.

#### DISCUSSION

The collection of 158 strains of *Vibrio cholerae* examined in this study comprised non-exacting strains capable of growing on ammonia and glucose as the sole sources of nitrogen and carbon, as well as exacting strains, which required purines for growth. The equal prevalence of the two in the present collection suggests that mutation from one to the other may be of high frequency. The

Table 3. *Growth of Vibrio cholerae and Salmonella typhimurium strains in minimal media containing heated animal sera*

Strain	Growth requirement	Sera added							Minimal medium (control)
		Human	Rabbit	Goat	Rat	Mouse	Guinea-pig	Horse	
			Growth on minimal medium	on minimal medium + 25 % (v/v) serum					
<i>V. cholerae</i>									
Ogawa 871	Purine	+	+	+	—	—	—	—	—
Ogawa 842	Purine	+	+	+	—	—	—	—	—
Inaba 791	Purine	+	+	+	—	—	—	—	—
Inaba 827	Purine	+	+	+	—	—	—	—	—
Inaba 8024	Non-exacting	+	+	+	+	+	+	+	+
Ogawa 502	Non-exacting	+	+	+	+	+	+	+	+
<i>S. typhimurium</i>									
Ad 2	Purine	+	+	+	—	—	—	—	—
Ad 10	Purine	+	+	+	—	—	—	—	—
Ad 2/p	Non-exacting	+	+	+	+	+	+	+	+
Ad 10/p	Non-exacting	+	+	+	+	+	+	+	+

—, no growth; +, growth.

uniform requirement for purines by the vast majority of exacting strains of *V. cholerae* is not paralleled in any other bacterial species whose nutritional requirements are known. There was no uniform difference in nutritional requirements between the antigenic types Inaba and Ogawa and Rough forms of *V. cholerae*. Both non-exacting and purine-dependent strains occurred in all three groups, and in individual Ogawa strains it was found that Inaba-type mutants had the same nutritional requirement as the parent strain.

It was also found that the sera of man, rabbit and goat were able to support the growth of purine-requiring strains whereas the sera of rat, mouse, guinea-pig and horse could not. It is of interest to note that two of the three sera which supported growth were those of man, to whom the organism is a natural pathogen, and rabbit, in which young animals are susceptible to experimental infection simulating cholera (Dutta & Habbu, 1955). Perhaps the administration of purines, either parenterally or orally, to refractory laboratory animals such as mice and guinea-pigs might increase their concentration in the sera of these animals and so induce susceptibility to *Vibrio cholerae*.

Tests with known purine antagonists (6-mercaptapurine, 8-azaguanine) showed that the former compound inhibited the growth of *Vibrio cholerae*, both purine-dependent and non-exacting, in purine-containing media, when the relative concentration in the medium of purines and antagonist was carefully adjusted. This finding is being further investigated. It is possible that such inhibitory compounds may prove to be of therapeutic value in the treatment of cholera.

The requirement of purines by strains of *Vibrio cholerae* might be applied to laboratory procedures for isolating cholera vibrios by supplementing culture media with purines, or by substituting purines in the place of complex protein digests. By the latter procedure contaminant flora might be restricted, and so permit the omission of suppressive substrates which are sometimes inhibitory even to *V. cholerae* itself.

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## A Comparison of the Staining Reactions of the Cell Walls of *Azotobacter chroococcum* and those of Gram-positive and Gram-negative Bacteria

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**SUMMARY:** The effects of various reagents in the mordanting and staining of bacterial cell walls are described. The cell walls of Gram-positive bacteria were found to be much more readily stainable than those of Gram-negative organisms. In this and other respects, apart from the Gram reaction *Azotobacter chroococcum* resembled a Gram-positive species; some of the methods described provided an excellent illustration of its *Bacillus*-like morphology.

The cell walls of bacteria may be stained by the use of mordants such as tannic acid (Eisenberg, 1910) or phosphomolybdic acid (Hale, 1953) followed by basic dyes, by complexes of dyes such as the fuchsin-congo-red method of Chance (1953), and certain of the methods of Yoshida *et al.* (1954), and by such rather exceptional dyes as Alcian blue (Tomesik & Grace, 1955) which stain the cell wall directly without the intervention of a mordant. The dye-complex methods are open to the criticism of Girbardt & Taubeneck (1955) that they do not so much stain the wall as flocculate against it. Accordingly, in the following investigation these were avoided, and the effects of a range of compounds employed as mordants upon the cell walls of a variety of bacteria were examined and compared. In addition to Alcian blue, it was also found that Janus green would stain unmordanted cell walls, and the comparison was extended to these dyes.

Since it has frequently been observed that not all bacteria react in the same manner to such staining procedures, it was considered necessary to make these tests upon a representative selection of Gram-positive and Gram-negative bacteria. And at the same time these were compared with *Azotobacter*, which, as has already been suggested (Bisset, 1955), although normally Gram-negative, possesses numerous characters suggestive of a relationship with the Bacillaceae.

### METHODS

The bacteria examined were one strain each of *Bacillus cereus* and *B. subtilis*, *Staphylococcus albus*, *Mycobacterium lacticola*, a large coccus of *Sarcina* type, *Aerobacter aerogenes*, *Escherichia coli*, *Proteus vulgaris*, and three of *Azotobacter chroococcum*. All were freshly isolated in this laboratory, with the exception of *Mycobacterium lacticola* which was a stock culture.

The *Azotobacter* strains were isolated on the nitrogen-free mannitol-phosphate medium described by Bisset & Hale (1953), on which they grew as a tough pellicle, composed of Gram-negative, almost coccoid, capsulated organisms,

entirely typical of the genus and species. For purposes of examination, however, after purification upon a similar medium, solidified with 2 % agar, they were grown upon potato-meal agar, which encourages their growth and spore formation (Bisset, 1955). *Mycobacterium lacticola* was grown on Lowenstein-Jensen medium, the remainder on heart-infusion agar. Parasitic forms were incubated at 37°, saprophytes at 30°.

Smears were made, rather thickly and without the addition of water, upon no. 1 cover-glasses, and air-dried for a few seconds before being placed in the mordanting solution. After this treatment they were washed in tap water and stained, usually with a 1 % aqueous solution of crystal violet, methyl green or thionin. Some of the original thick smear was washed off in the course of these procedures, leaving a thinner residue firmly adhering to the glass. When simple staining methods were used, without previous mordanting, for example with Alcian blue or Janus green, thinner smears were made in the first place. The stained preparations were mounted in water by sealing the cover-glass to a slide at the edges with melted beeswax.

In Table 1 are listed the reagents used as mordants. They were applied as 1 % (w/v) aqueous solutions in every case, and the subsequent staining was with crystal violet, which, by comparison with methyl green and thionin, was found

Table 1. *The effects of various staining methods upon the cell walls of bacteria. The mordants were applied as 1 % aqueous solutions, and subsequent staining was with crystal violet*

Mordants	<i>Bacillus</i> species	<i>Mycobacterium</i> <i>lacticola</i>	Cocci	Bac- teriaceae	Azoto- bacter strains
Sodium bicarbonate	—	±	+	—	±
Sodium lactate	±	—	—	—	±
Sodium nitrate	±	—	—	—	—
Sodium chloride	—	—	±	—	—
Sodium hyposulphite	—	+	+	—	—
Potassium tellurite	—	±	+	—	±
Potassium hydroxide	±	±	±	—	+
Calcium hydroxide	+	+	+	±	+
Lithium carbonate	—	±	+	—	—
Ferric alum	—	—	+	—	—
Ammonium alum	—	—	+	—	±
Copper sulphate	—	—	±	—	±
Phosphomolybdic acid	+	+	+	±	+
Phosphotungstic acid	+	±	±	±	±
Acetic acid	—	—	—	—	±
Oxalic acid	—	—	—	—	±
Lactic acid	—	—	—	—	±
Tannic acid	+	+	+	±	+
Stearic acid	—	—	±	—	—
Phenol	—	±	±	—	—
Tween 80	—	+	±	—	—
Dyes					
Alcian blue	±	±	+	±	+
Janus green	+	+	+	±	+

+ = stains well regularly; ± = stains irregularly; — = fails to stain.

to be most generally satisfactory for the purpose, although the other dyes gave better results occasionally. Mordanting and staining were both conducted at room temperature. Higher temperatures and stronger solutions were used experimentally, but these conferred no apparent advantage. Preparations were mordanted for *c.* 15 min. and stained for *c.* 2 min. in every case. Substances which gave uniformly negative results are not listed.

Janus green (Hopkins & Williams) and Alcian blue (Gurr) were kept at 1 % (w/v) solutions in 40 % (v/v) ethanol in water, and diluted with distilled water for use. In the case of Janus green a period of 5 min. in a 0.01 % solution produced the best results. In overstained preparations the dye diffused out into the mountant. Alcian blue was used at 0.1 % for 15–30 min., being a much weaker dye.

Preparations were made from 18 hr. cultures, in order to obtain active vegetative forms. However the cocci stained well at any stage of cultivation.

### RESULTS

In Table 1 are shown the results of cell-wall staining, using as mordants the reagents listed in the vertical column. It can be observed that most Gram-negative bacteria stained much less well than did the Gram-positive organisms, but that the azotobacters stained exceptionally well. Some of the methods used gave an unusually good illustration of the cytological structure of *Azotobacter chroococcum*, and are illustrated in Pl. 1. Calcium hydroxide not only acted as a mordant but when heated to 100° it partially plasmolysed the cell contents and revealed the complexities of the structure very clearly (see Robinow, 1945, who used boiling sodium hydroxide solution). Some rods appeared as almost empty cell walls with well-marked cross-walls (Pl. 1, figs. 1, 2). Others showed differentially plasmolysed protoplasts, representing cells which had occupied a quarter, a half or the entire rod. Thus varying degrees of cellular complexity were illustrated (Pl. 1, figs. 3–6). The appearance of these plasmolysed cells and cell walls was very *Bacillus*-like. Potassium hydroxide produced a less marked effect, and failed to react with the Gram-negative bacteria, apart from *A. chroococcum*.

A second method which provided a clear illustration of the *Bacillus*-like characters of *Azotobacter chroococcum* was Janus green, which served to demonstrate not only the cell walls but also the 'cyst walls' (i.e. the spore coats) of the 'encysting' cultures. The endospore-like origin of the 'cysts', and their release by rupture of the sporangium wall were both clearly seen (Pl. 1, figs. 7, 8).

It is remarkable that although the cocci and the azotobacters were both exceptionally easy to stain, they did not react to the same compounds. For example, the three low-molecular-weight organic acids, acetic, oxalic and lactic acids, were effective only with the *Azotobacter* strains, of all the organisms examined. The same applied to such diverse compounds as ferric alum, stearic acid and even sodium chloride, in the case of the cocci. No explanation can be offered for these phenomena. In the cases of the metallic salts and the

organic compounds of higher molecular weight, the reactions of the *Bacillus* species often differed from those of the other Gram-positive organisms, and sometimes then agreed with those of the *Azotobacter* strains.

#### DISCUSSION

Comparisons between the efficacy of different compounds as mordants gives very little help in the elucidation of the problem of what the mordanting process in bacterial cell-wall staining actually entails. The reactions observed vary very considerably, and quite unpredictably, from genus to genus, and especially as between Gram-positive and Gram-negative bacteria. The cell walls of the latter are much less easy to demonstrate. In this, as in certain morphological respects, *Azotobacter chroococcum* behaves like a Gram-positive organism, although normally staining Gram-negatively; some of the methods used provide an excellent illustration of the morphology of this rather curious organism.

Many previous investigations of cell-wall staining have been made upon single subjects, usually species of *Bacillus* (e.g. Yoshida *et al.* 1954), with the tacit assumption that all other bacteria react similarly, which is now seen to be quite unjustifiable. The best-known methods, using tannic or phosphomolybdic acid, have been successful precisely because their originators, including the present writers in the latter case, experimented with a variety of different bacteria, and found them all to react satisfactorily. Next best after these are probably the dyes Alcian blue and Janus green, which stain without previous mordanting.

Such little evidence as we possess about the comparative chemical constitution of bacterial cell walls suggests that Gram-positive and Gram-negative organisms are distinctly different in this respect (Salton, 1956); the anomalies recorded in this paper may be of interest to subsequent investigators in this field.

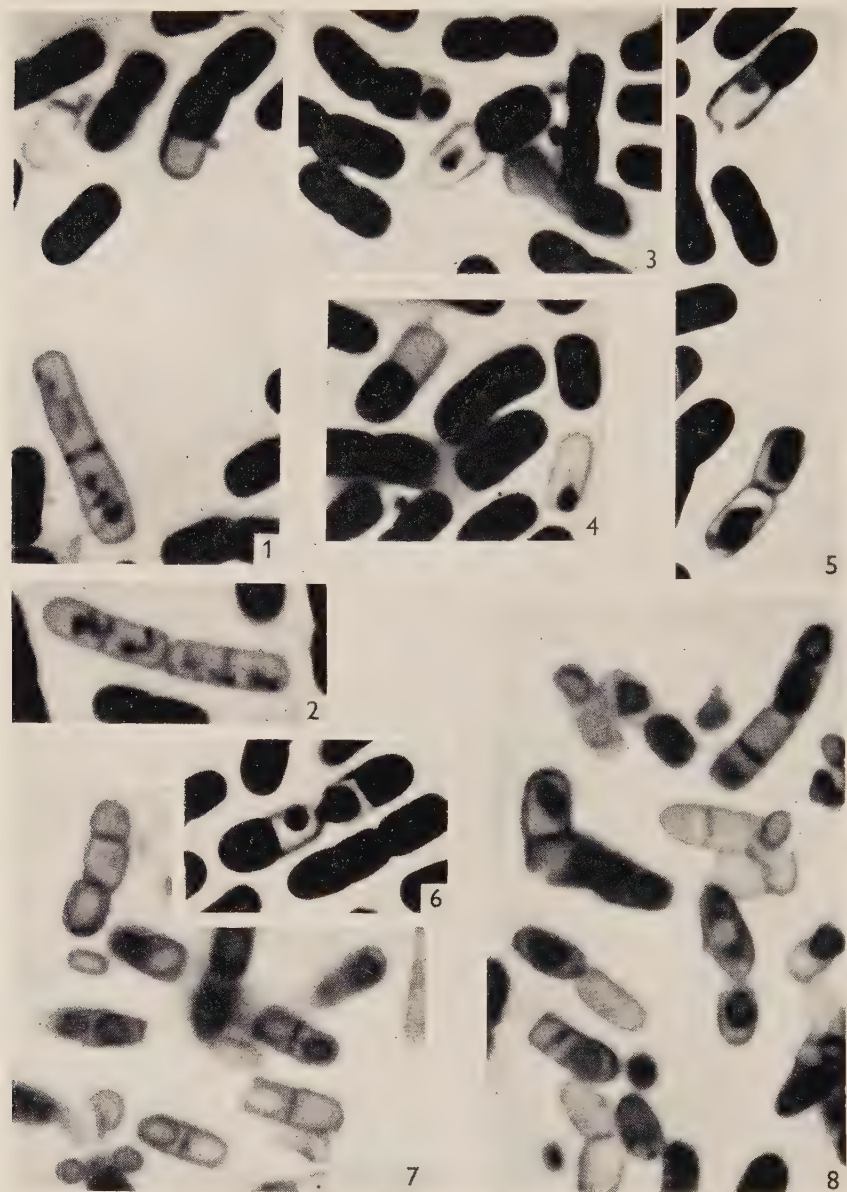
The evidence in respect of the *Azotobacter* strains used tends to confirm the previous observations which suggest a common ancestry for aerobic and anaerobic nitrogen-fixing bacteria (Bisset, 1955), and is in accordance with the occasional reports of early investigators that strains of this genus may be found to stain Gram-positively. The nature and behaviour of such strains will be the subject of a further communication.

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C. M. F. HALE AND K. A. BISSET—STAINING REACTIONS OF CELL WALLS. PLATE 1  
(Facing p. 427)

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#### EXPLANATION OF PLATE

- All figures are of *Azotobacter chroococcum*, grown on potato-meal agar.  $\times 3000$ .
- Figs. 1-6. Young culture (24 hr.) showing varying degrees of plasmolysis by calcium hydroxide solution. Septate filaments and bacilli containing one, two and four cells are shown.
- Figs. 7-8. Sporulating culture (1 week old) stained with Janus green to show cell walls and spores; some of the latter in process of ejection.

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## Comparison of Physiological and Biochemical Characters of *Actinomyces* spp. with those of *Lactobacillus bifidus*

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**SUMMARY:** Evidence is presented that under the conditions described 11 strains of *Actinomyces* spp., representing strains described as *A. israelii*, *A. israelii*-like or *A. naeslundii*, require carbon dioxide for anaerobic growth. Some of these strains, under these conditions, are obligate anaerobes to microaerophils, while others appear to be facultative anaerobes. Cultures which are capable of aerobic growth may or may not require carbon dioxide for such growth. Of three strains of *Lactobacillus bifidus* tested, all required carbon dioxide for anaerobic growth. One avian strain required carbon dioxide to give limited aerobic growth; the remaining strains did not grow significantly under aerobic conditions. Comparisons of several strains of *Actinomyces* spp. with *L. bifidus* indicated that of eleven sugars tested, the sugar of choice for growth of *Actinomyces* spp. was glucose or maltose, whereas lactose or maltose was preferred by strains of *L. bifidus*. All strains of each group of organisms were found to be catalase-negative; none liquefied gelatin; all eleven strains of *Actinomyces* spp. reduced nitrate to nitrite, but none of the bifid strains possessed this ability; production of acetylmethylcarbinol was variable in both groups. All strains of *Actinomyces* spp. tested formed L (+) lactic acid, although the results suggested that small amounts of D (-) lactic acid were also formed. Fermentation analyses indicated that strains of *L. bifidus* and *Actinomyces* spp. form the same products from glucose and carbon dioxide (lactic, acetic, formic and succinic acids). However, strains of *Actinomyces* spp. form predominantly lactic acid with small amounts of acetic, formic and succinic acids; whereas the strains of *L. bifidus* form approximately equal amounts of lactic and acetic acids (based on glucose fermented) with trace amounts of succinic and formic acids. *Actinomyces* strains fermented but 34-59 % of the glucose supplied as compared to the strains of *L. bifidus* which used from 59 to 89 % of the glucose (1 % glucose medium).

The relationship of *Lactobacillus bifidus* to the genus *Actinomyces* was apparently first noted by Vuillemin (1931). Puntoni (1937) noted certain morphological, biochemical and immunological resemblances between strains of *Actinomyces* spp. and *L. bifidus*. He concluded that the organisms were sufficiently similar to be placed in the same genus and species, although he felt a species differentiation might be made. Negroni & Fischer (1944), from a study of *L. bifidus*, concluded that this organism should be placed in the genus *Cohnistreptothrix* (*Actinomyces*). Frank & Skinner (1954), on primarily morphological grounds, suggested that *L. bifidus* be placed in the genus *Actinomyces* as *A. bifidus*. However, each of these studies was somewhat limited in extent. In addition,



any conclusions regarding the relationship of these organisms to one another have been limited by the lack of information on certain biochemical properties of organisms of the genus *Actinomyces*. Consequently, it seemed worth while to make comparisons of these two groups of organisms on the basis of: (1) their ability to grow aerobically or anaerobically, with and without added carbon dioxide; (2) their ability to utilize various sugars; (3) their fermentation products from glucose; (4) certain physiological properties. The results of these experiments are presented here.

## METHODS

### *Strains of organisms*

Twelve strains of *Actinomyces* spp. and four of *Lactobacillus bifidus* were studied. The strains of *Actinomyces* spp. were those described previously (Howell & Pine, 1956), except that the bovine isolates, strains 278 and 284, were not included in the present study. Cultures 306 and 307 were avian strains of *L. bifidus* obtained through the courtesy of Mr Morrison Rogosa, National Institute of Dental Research. Strains 308 and 309 were human isolates of *L. bifidus* described as 'Timberlaine' and 'Jackson' strains, respectively, by Norris, Flanders, Tomarelli & György (1950), and were obtained from Dr Paul György, Department of Pediatrics, University of Pennsylvania Hospital. Cultures of *Actinomyces* spp. were maintained as described previously (Howell & Pine, 1956); those of *L. bifidus* were maintained on the acetate medium of Norris *et al.* (1950) modified by the omission of pancreatin and sorbitan mono-oleate as recommended by Norris (personal communication).

### *General methods*

The procedures for the growth and preparation of inoculum for all cultures and measurements of growth were those described earlier (Howell & Pine, 1956). To study growth anaerobically in the absence of carbon dioxide, a pyrogallol + KOH (10 %, w/v) seal was used; to measure growth aerobically in the presence of increased carbon dioxide, a 10 % (w/v)  $\text{Na}_2\text{CO}_3$  +  $\text{m-KH}_2\text{PO}_4$  seal was used. In either case five drops of each reagent were used per growth tube. For studies on growth in air, the cotton plug and rubber stopper were replaced by an aluminium cap. In the experiments in which oxygen tolerance or carbon dioxide requirements were studied, all cultures were incubated at 37° on a rotary shaker as described previously (Howell & Pine, 1956). With the conditions of shaking under which such cultures were incubated in the presence or absence of added carbon dioxide, methylene blue (2 drops of a  $10^{-4}$  dilution/5 ml. medium) was not reduced. In those cultures in which growth was obtained in the presence of oxygen, serial transfers were made when the optical density reached 0.500 or greater. For other studies anaerobic cultures were incubated stagnant inasmuch as shaking did not appear significantly to affect growth of actinomycetes (Howell & Pine, 1956).

Unless stated otherwise, the medium used for all comparative growth experiments was the liquid defined medium with starch and Casitone described

by Howell & Pine (1956) containing 0.5 % (w/v) sugar. For fermentation analyses the same medium with 0.5 or 1.0 % (w/v) glucose and either casein hydrolysate, Casitone, or Casitone supplemented with 0.5 % (w/v) yeast extract was used. The inoculum was grown anaerobically in the 0.5 % (w/v) glucose Casitone medium.

*Cultural methods for fermentation analyses*

As reported previously, several of the *Actinomyces* strains show poor growth at pH 7.0 in the casein hydrolysate medium, although excellent growth is obtained at pH 6.5. Consequently, in cases where accurate carbon dioxide

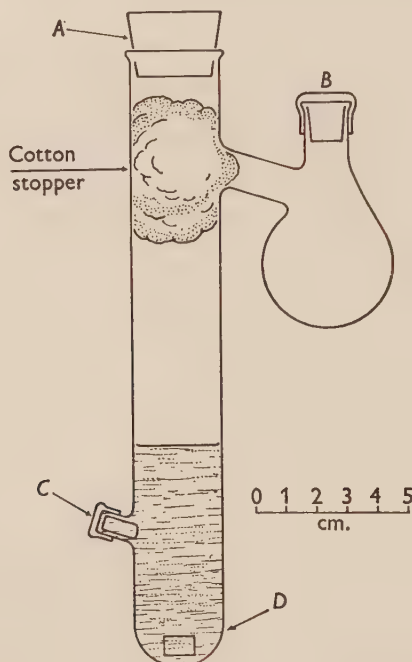


Fig. 1. Diagram of fermentation tube used for carbon dioxide analyses.

determinations were to be made a system was necessary which would allow the addition of gaseous carbon dioxide at pH 6.5 and analyses of residual carbon dioxide without loss to, or contamination from, the air. Initially, 100 ml. volumetric flasks filled to the mark were sealed with sterile vaccine bottle stoppers. By the use of hypodermic needles pushed through the stoppers, the air phase was replaced with a 95 %  $N_2$  + 5 %  $CO_2$  (v/v) mixture. When growth had apparently ceased, carbon dioxide-free NaOH was injected through the stopper until the medium was alkaline, and a sample was then withdrawn for analysis. As will be described later, such flasks, although usable, were not entirely satisfactory. Good growth was more consistently obtained with the type of tube shown in Fig. 1, containing 25–30 ml. of medium. In this case, after the medium was inoculated, the cotton stopper, used in autoclaving before

the introduction of the medium, was pushed into the tube to block the side arm orifice and the tube was sealed with a no. 4 rubber stopper, *A*. A no. 20 hypodermic needle attached by a rubber hose to a vacuum pump was then pushed through the vaccine stopper, *B*, and the system evacuated for 1–2 min. The needle was withdrawn and 3 ml. of a pyrogallol +  $\text{H}_2\text{SO}_4$  solution (30 g. pyrogallol dissolved in and made to 100 ml. with  $\text{N-H}_2\text{SO}_4$ ) were introduced with a syringe into the side bulb through *B*. Next 4 ml. of 10 %  $\text{Na}_2\text{CO}_3$  (w/v) solution were injected slowly into the bulb. The cotton stopper prevents contamination by vapour formed by the release of carbon dioxide. At the end of the growth period sufficient carbon dioxide-free NaOH to make the medium pH 9 or greater was injected directly into the medium through the stopper at *C*. The tube was incubated overnight or longer at 5° to absorb the carbon dioxide into the medium. One ml. samples were then withdrawn with an Ostwald-Van Slyke pipette with stopcock and rubber tip for determination of the final carbon dioxide content of the medium. In this type of tube the granules of *Actinomyces* strains rapidly settled to the bottom and growth was localized there. Therefore a magnet, *D*, was used in conjunction with a magnetic stirrer to mix the culture sediment throughout the medium several times each day. Uninoculated controls were used to determine the initial carbon dioxide concentration. In one experiment four replicate determinations of the initial carbon dioxide present in uninoculated controls gave values of 25.62, 24.25, 26.71, and 24.73  $\mu\text{mole/ml}$ .

In some cases, where carbon dioxide was not determined, cultures were grown in 25 × 200 mm. test tubes containing 25 or 30 ml. of medium with a pyrogallol +  $\text{Na}_2\text{CO}_3$  seal.

#### Chemical methods

Nitrate reduction was tested on horse meat infusion broth containing 1 % trypticase, 0.5 % NaCl and 0.1 %  $\text{KNO}_3$ . Qualitative nitrite determinations were made daily for 7 days (*Manual of Methods for Pure Culture Study of Bacteria*, 1946); residual nitrate was determined after 7 days incubation. Gelatin liquefaction was tested after 10 days incubation on the horse meat infusion broth containing 10 % (w/v) gelatin. Cultures on litmus milk were incubated for 10 days. Catalase was determined on colonies of actinomycetes grown for 7 days on brain heart infusion agar plates by adding 1–2 drops of a freshly prepared 10 % (v/v) solution of Superoxol (Merck and Co., Reagent, hydrogen peroxide 30 %) and observing gas formation. Cells of *Candida albicans* served as a positive control. All cultures for determination of nitrate reduction, gelatin liquefaction, action on litmus milk and catalase reaction were incubated anaerobically in Brewer jars with 95 %  $\text{N}_2$  + 5 %  $\text{CO}_2$  at 37°.

Carbon dioxide was measured by the method of Peters & Van Slyke (1932) or with the Warburg apparatus. Initial and residual glucose were determined by the method given by Umbreit, Burris & Stauffer (1949). Ethanol was identified qualitatively by diffusion into dichromate (Winnick, 1942) and the acid formed by its Duclaux value. Quantitative determinations of acetoin were made by the procedure of Westerfeld (1945). Quantitative estimation of

glycerol in several fermentations by the procedure of Smith (1950) showed no significant formation of this compound. Tests for organic compounds not mentioned above were made by the dichromate oxidation procedure of Johnson (1949). Formic and acetic acids were identified qualitatively as a mixture by their Duclaux values and were then identified by paper chromatography and  $R_f$  values by the procedure of Kennedy & Barker (1951). Formate was also shown to be present at the correct  $R_f$  by spraying with 5% (w/v) ammoniacal  $\text{AgNO}_3$ . Formic and acetic acids formed by strains of *Actinomyces* spp. were then determined quantitatively by the procedure of Friedemann (1938) as modified by Rabinowitz & Barker (1956). Formic acid formed by strains of *Lactobacillus bifidus* in the acetate medium (Norris *et al.* 1950) containing glucose or lactose, was identified and estimated in acid steam distillate by the formic acid hydrogenlyase method of Gest (1952). Four ml. Warburg vessels were used. It was estimated in other media by the procedure of Rabinowitz & Barker (1956).

Succinic acid was identified as a product of glucose fermentation by actinomycetes by fermenting glucose- $^{14}\text{C}$  with strains 279 and 295. Isolation of an unknown radioactive non-volatile acid was accomplished by paper chromatography (Block, Durrum & Zweig, 1955) and subsequent radioautographs. Its identity was indicated by a dilution and spreading of the radioactive spot upon the addition of unlabelled succinic acid to paper chromatograms. Ethanol + ammonia (Kennedy & Barker, 1951) and methanol + pyridine (Redfield, 1953) were used as solvents. The unknown compound was separated from lactic acid by the procedure of Phares, Mosbach, Denison & Carson (1952). Titration values and radioactivity determinations on the effluents of a Celite column showed a direct correspondence of the peak with known succinic acid. Separation from lactic acid was also accomplished by eluting the radioactive strip from paper chromatograms. The separated succinic acid fractions were assayed by the succinoxidase procedure of Umbreit *et al.* (1949) using 5 ml. Warburg vessels. Subsequently, fermentation analyses for succinic acid were made directly on the non-volatile ether-extractible acid fraction. Lactic acid did not interfere with the determination.

Lactic acid was isolated and identified as the zinc salt according to the procedure of Pederson, Peterson & Fred (1926). It was determined quantitatively by the method of Barker & Summerson (1941). The oxidation-reduction indices (redox values) were calculated according to the method of Neish (1950).

## RESULTS

### *Comparison of media for growth of Lactobacillus bifidus*

Preliminary experiments indicated that the rates of growth of each of the four strains of *Lactobacillus bifidus* in the liquid medium with starch (Casitone and 0.5% lactose; Howell & Pine, 1956) were essentially comparable to those obtained in the acetate lactose medium of Norris *et al.* (1950) (Fig. 2). Furthermore, although the amount of growth obtained with the human bifid strains was less than that obtained on the lactose medium of Norris *et al.*



(1950), the amount of growth of the avian strains was significantly greater on the lactose Casitone starch medium than on the lactose medium. Gram stains of bifid cultures 308 and 309 on the former medium showed more 'straight rod' forms than were usually obtained on the medium. However, subcultures on to the lactose agar of Norris *et al.* (1950) from cultures of strains 308 and 309 on the lactose Casitone starch medium, made after maximum growth was obtained, yielded only the typical 'bifid type' colonies described by Norris *et al.* (1950). Gram stained films of these colonies showed consistently and almost exclusively bifid morphology. Similar results were obtained when glucose was substituted for lactose in the Casitone medium, with the exception that strain 306 failed to grow on Casitone glucose. Since the casitone starch medium appeared to be a satisfactory medium for the growth of all four strains of *L. bifidus*, did not induce 'mutations' in these cultures, and had previously been shown to support excellent growth of all strains of *Actinomyces* spp., it was chosen as the basic medium for the comparative study of cultures of *L. bifidus* and *Actinomyces*.

*Oxygen tolerance and carbon dioxide requirements of strains of Actinomyces spp. and Lactobacillus bifidus*

From Table 1, it is seen that all cultures of *Actinomyces* spp. and *Lactobacillus bifidus* required carbon dioxide for anaerobic growth in the liquid glucose Casitone starch medium. Four of the six *Actinomyces* capable of aerobic growth under these conditions and the single strain of *L. bifidus* capable of limited aerobic growth required carbon dioxide for such growth. Subsequent experiments, however, indicated that growth of strain 296 in air without carbon dioxide, and of strain 282 in air + carbon dioxide was not a constant finding.

Of the *Actinomyces* capable of aerobic growth on the first transfer, five appeared capable of continuous transfer in air of increased carbon dioxide tension. These cultures gave as much or more (in several instances twice as much) growth aerobically as they did anaerobically with carbon dioxide (Table 1). In those cultures grown aerobically or in air plus carbon dioxide on which a final pH value was determined, the terminal pH value was found to be approximately 5.0–6.1 as compared to about 4.5 usually obtained in cultures grown anaerobically. Strains 279, 282 and 296 showed no

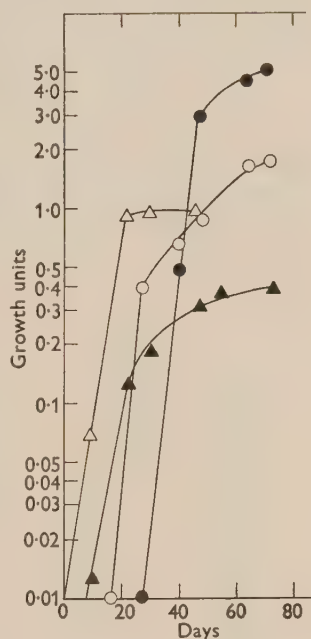


Fig. 2. Rate of growth of human (308) and avian (307) strains of *Lactobacillus bifidus* in the lactose medium of Norris *et al.* (1950) and on the lactose-Casitone medium. Strain 307: ▲, △; strain 308: ●, ○; ▲, ● = 1.75% lactose, medium; △, ○ = 0.5% lactose, Casitone medium.

decrease in rate or amount of anaerobic growth after four to six consecutive aerobic transfers (with added carbon dioxide). These cultures, particularly those which grew aerobically, were examined by Gram reaction and by wet mounts made at the period of maximum growth. In no instance in the reported experiments was there observed any indication of contamination or gross change in morphology. In other experiments (Howell, unpublished), colonies of strains 279 and 296 developed on plates of this medium containing 1.5 % (w/v) agar in air+5 % (v/v) carbon dioxide. These colonies, though

Table 1. *Amount of growth of strains of Actinomyces spp. and Lactobacillus bifidus obtained anaerobically and aerobically in the presence and absence of added carbon dioxide*

	Anaerobic		Aerobic*	
	-CO <sub>2</sub>	+CO <sub>2</sub>	-CO <sub>2</sub>	+CO <sub>2</sub>
	Maximal growth units obtained			
<i>Actinomyces</i> spp.				
261	None	2.90	0.10 (1)	2.68 (1)
262	None	1.83	0.14 (1)	0.12 (1)
263	None	2.25	0.10 (1)	3.23 (5)
279	None	3.00	3.60 (5)	5.60 (5)
281	None	2.32	0.11 (1)	0.47 (1)
282	None	2.75	0.07 (1)	4.40 (4)
283	None	1.60	0.02 (1)	0.06 (1)
286	None	3.50	0.13 (1)	6.20 (6)
287	None	0.80	0.13 (1)	0.13 (1)
295	None	2.63	0.20 (1)	0.52 (1)
296	None	2.35	0.71 (2)	4.48 (5)
<i>L. bifidus</i>				
307	None	1.40	0.00	0.70 (1)
308	None	3.11	0.00	0.12 (1)
309	None	3.17	0.00	0.00

\* The numbers in parentheses indicate the number of consecutive transfers in which growth was obtained and the maximum growth (expressed as growth units) obtained on the first transfer.

fewer in number than on similar plates incubated in an atmosphere of 5 % CO<sub>2</sub>+95 % N<sub>2</sub> (v/v) were identical in colony morphology with those developed on the plates incubated anaerobically. Similar studies (Pine, unpublished) with strain 296 using large agar slants and both the potassium phosphate+sodium carbonate, or pyrogallol+sodium carbonate seals described above, gave identical colonies and cell morphology. From these results, and those presented in Table 1, it would appear that under these conditions some strains of the genus *Actinomyces* are facultative anaerobes, whereas others are obligate anaerobes or microaerophils.

A single strain of *Lactobacillus bifidus* (307) showed some aerobic growth in the presence of added carbon dioxide, but the amount was but half that obtained anaerobically with carbon dioxide and did not continue on the second transfer (Table 1). The other two lactobacillus cultures showed no aerobic growth. These cultures would appear to be anaerobes or microaerophils.

Cultures of *Lactobacillus bifidus* were also tested for an anaerobic carbon dioxide requirement on the same medium with 0.5 % (w/v) lactose. Of the four strains tested, only the avian strains (306 and 307) grew anaerobically on lactose without added carbon dioxide. However, only half the amount of growth was obtained under these conditions as was obtained on this medium with added carbon dioxide. It would appear, therefore, that with *L. bifidus* the medium used may affect the requirement for carbon dioxide.

*Growth of Lactobacillus bifidus and Actinomyces spp. on sugars*

Rose, Luchi, Avery, Norris & György (1953) reported that human strains of *Lactobacillus bifidus* utilized the disaccharides lactose and maltose more readily than glucose and implied that this finding might be of value in differentiating *L. bifidus* from its straight rod variants and *L. acidophilus*. Comparisons were

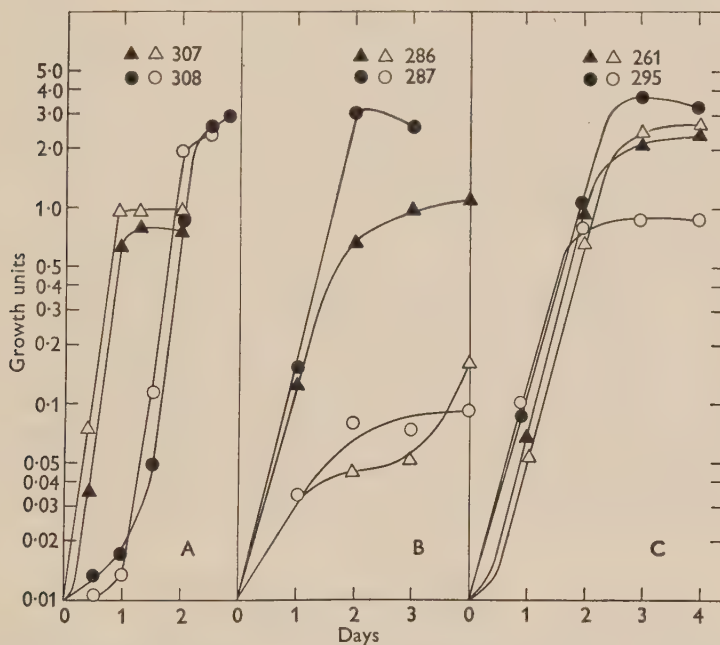


Fig. 3. Rate of growth of strains of *Lactobacillus bifidus* and *Actinomyces* spp. on glucose and lactose. A: *L. bifidus*; B, C: *Actinomyces* spp.; ●, ▲ = glucose; ○, △ = lactose.

made, therefore, of the relative utilization of these three sugars by strains of *L. bifidus* and the actinomycetes to determine whether or not these two groups of organisms behaved similarly under these conditions.

Under the conditions of this experiment, it was found that the rates and amounts of growth of all lactobacilli tested were, in general, similar on lactose and glucose except for strain 306 which did not grow on glucose (Fig. 3A). In some instances the rates of growth were slightly higher on lactose but these results were not consistent. However, growth on glucose was never significantly greater than on lactose. In a single experiment, rates

of growth of strains 307, 308 and 309 were slightly higher on maltose than on glucose but the difference was not regarded as significant. Strain 306 was not tested on maltose.

On the other hand, most cultures of the *Actinomyces* were more 'active' on glucose than on lactose. One group of strains (277, 279, 286 and 287) grew more slowly and erratically with lactose as a substrate than with glucose and the final amounts of growth were much greater with glucose (Fig. 3B). A second group (strains 295 and 296) showed equal rates of growth on the two sugars but the amount of growth on glucose was three to four times that obtained on lactose (Fig. 3C). Other strains (261, 263, 282 and 283) showed equal rates and amounts of growth on the two sugars (Fig. 3C). In no instance, however, was growth on lactose considered to be superior to that on glucose. Results with maltose were essentially identical with those on glucose with each of the ten strains tested.

The relative ability of these two groups of organisms (except strain 306) to utilize other carbohydrates and pyruvate and gluconate as carbon sources was then tested. Carbohydrates employed were arabinose, mannitol, mannose, raffinose, rhamnose, ribose, salicin and sucrose. In general, no single one of these substrates showed a clear differentiation of lactobacilli as a group from the actinomycetes as a group (Table 2). Little or no growth of either group was obtained on the basal medium alone.

In addition to the results reported above, the actinomycetes and *Lactobacillus bifidus* were tested for the presence or absence of certain other biological properties. All strains of each group of organisms were found to be catalase-negative; none liquefied gelatin; all eleven strains of *Actinomyces* spp. were found to reduce nitrates to nitrites, but none of the lactobacilli strains possessed this ability; production of acetylmethylcarbinol was variable in both groups, apparently being dependent on the medium; and finally, both groups showed similar activity on litmus milk in that, in general, they produced acid and showed some reduction, both properties varying in extent, dependent on the strain.

#### *Studies on the fermentation of glucose by Actinomyces and Lactobacillus bifidus*

The results of analyses of fermentations of seven *Actinomyces* strains on 1% (w/v) glucose in the basal medium prepared with casein hydrolysate are given in Table 3. In this medium and under the conditions employed, c. 90–100% of the carbon of the glucose fermented by these strains was determined as lactic acid. All cultures, however, produced small amounts of formic and acetic acids (3.5–11.8% of the glucose fermented). A compound tentatively identified as ethanol accounted for a maximum of 2% of the glucose fermented. Although acetylmethylcarbinol was found in trace amounts in other media, none was formed in the fermentations presented in Table 3. Starch, which is present in the medium at a concentration of 0.05% (w/v), is known to support a small amount of growth of at least strains 277, 279, 286 and 287; its presence is accounted for in the calculations of percentage



Table 2. Maximum amount of growth\* obtained on specified substrates with strains of *Lactobacillus bifidus* incubated 10 days and *Actinomyces* spp. incubated for 7 days

Strain	Substrate										
	Glucose	Lactose	Maltose	Sucrose	Mannitol	Rhamnose	Arabinose	Raffinose	Salicin	Ribose	Mannose
<i>Actinomyces</i> spp.											
261	2.44	2.70	1.82	1.06	2.25	0.24	0.99	2.92	1.18	2.40	1.95
263	3.20	2.12	1.85	—	0.14	0.12	0.19	—	—	—	—
277	2.41	0.59	2.87	0.82	1.90	0.32	0.62	—	—	—	—
279	3.60	0.42	3.28	—	0.14	0.26	0.23	2.22	1.62	—	0.95
281	—	—	—	—	—	0.12	0.40	0.53	0.66	0.60	0.97
282	3.00	2.80	4.10	—	1.34	0.50	0.40	1.35	1.29	1.88	1.09
283	2.64	2.32	3.77	3.33	2.23	0.52	0.19	2.72	2.10	1.51	1.09
286	2.95	0.28	3.04	3.78	0.20	0.19	0.14	2.02	0.80	0.08	0.88
287	2.32	1.47	2.17	0.78	0.68	0.18	0.29	0.60	0.73	0.78	0.53
295	3.78	0.90	2.57	2.88	2.30	0.36	0.61	0.76	0.97	0.76	0.43
296	3.00	0.98	1.14	—	1.19	0.43	1.75	0.66	0.99	2.12	0.76
<i>L. bifidus</i>											
307	1.04	0.64	0.50	0.46	0.03	0.06	0.84	1.75	2.08	0.59	0.62
308	3.03	3.15	2.55	2.95	0.08	0.09	0.16	3.20	0.05	0.69	0.56
309	3.72	3.00	2.85	2.77	0.04	0.06	0.07	2.52	0.06	1.19	0.45

\* Readings taken daily and expressed as growth units (optical density  $\times 1/\text{dil.}$ ).

carbon recovered. In general carbon recoveries in this medium were high. The values obtained for carbon dioxide are questionable since the determinations employed the Warburg apparatus and were susceptible to contamination with carbon dioxide from the air. No fermentation analysis gave a satisfactory redox balance. Because of the small concentration of reduced or oxidized products, this was not considered important.

Table 3. *Analysis of glucose (1.0 %, w/v) fermentations by strains of Actinomyces spp. in basal medium with casein hydrolysate in 100 ml. volumetric flasks filled to the mark, and flushed 1-2 min. with 95 % N<sub>2</sub>-5 % CO<sub>2</sub> to remove air*

	Strain number						
	277	279	281	282	286	287	295
	$\mu\text{mole/ml.}$						
Glucose fermented	32.4	27.3	20.6	29.1	30.1	23.5	19.0
Products							
Carbon dioxide	0.2	0.6	0.0	0.3	0.5	0.0	0.4
Formic acid	3.2	3.6	4.2	3.9	2.4	4.7	4.5
Acetic acid	1.7	1.7	2.1	1.7	0.7	2.7	3.8
Lactic acid	64.4	53.2	41.0	57.1	58.5	42.3	38.1
Ethanol	—	1.2	—	—	1.5	1.8	0.7
Percentage carbon recovered*	101.5	98.8	103.6	99.7	98.6	96.4	108.3
Redox value	—	2.00	—	—	1.13	1.30	3.78
Percentage glucose fermented	57.9	49.1	37.0	52.4	54.2	42.4	34.2

\* Values of percentage carbon recovered have been corrected for the presence of 0.05 % (w/v) starch assuming utilization of the starch and weight to weight equivalence of starch to glucose.

In an attempt to determine whether or not the composition of the medium would significantly affect the products of glucose fermentations, three strains of *Actinomyces* were grown in the casein hydrolysate medium (1 %, w/v, glucose) and in the Casitone medium supplemented with 0.5 % (w/v) yeast extract, with and without 0.5 % (w/v) glucose. The results obtained are given in Table 4. A small amount of growth and fermentation occurred in the Casitone yeast-extract medium without glucose, but this growth was accounted for by the disappearance of reducing substances from the medium. Growth of these strains in the two sugar-containing media showed a wide diversity in the amount of lactic acid formed, the ratio of lactic acid to volatile acid formed, and in the ratio of formic to acetic acid formed. The amount of lactic acid formed in the casein hydrolysate medium was from 80 to 93 % of the glucose fermented. In the Casitone yeast-extract medium, lactic acid accounted for 63-82 % of the glucose fermented. Furthermore, carbon recoveries in the latter medium were significantly lower for two strains than that obtained in the casein hydrolysate medium.

In other experiments, carbon recoveries in the Casitone medium were generally lower than those obtained using casein hydrolysate. For example, analysis of fermentations of strains 277 and 282, in the Casitone medium with

Table 4. *Effect of the medium on the products of glucose fermentation by strains of Actinomyces spp. incubated in 25 x 200 mm. test tubes with pyrogallol-Na<sub>2</sub>CO<sub>3</sub> seal*

	Strain									
	279					295				
	Medium					Medium				
Glucose fermented Products	279					295				
	Casitone yeast extract* glucose†	Casitone yeast extract +0.5 % glucose†	Casein hydrolysate +1.0 % glucose	Casitone yeast extract* glucose†	Casein hydrolysate +1.0 % glucose	Casitone yeast extract +0.5 % glucose†	Casitone yeast extract +0.5 % glucose†	Casein hydrolysate +1.0 % glucose	Casitone yeast extract* glucose†	Casein hydrolysate +1.0 % glucose
	0.8	26.0	27.1	1.7	32.0	23.7	24.0	32.0	0.8	32.3
Formic acid	3.1	8.0	6.9	3.0	10.8	11.6	11.1	10.8	4.3	10.5
Acetic acid	4.6	2.2	1.5	3.1	4.9	8.5	9.4	4.9	3.1	5.3
Lactic acid	—2.4	31.9	44.1	0.5	57.0	29.9	39.8	57.0	0.8	59.9
Ethanol	0.2	0.0	0.2	0.8	1.0	0.0	0.0	1.0	0.7	0.6
Percentage carbon recovered	—	69.2	87.5	—	99.7	82.7	103.5	99.7	—	104.0

\* Net disappearance of initial reducing substances was calculated as glucose fermented.

† Values given are corrected for the values obtained in the absence of added sugar.

and without glucose, gave essentially the same results obtained with strain 295. Carbon recoveries in the presence of added sugar were 86 % in all cases. These results suggested that an unknown product was being formed. Subsequently, analyses of fermentations of glucose-<sup>14</sup>C with strains 279 and 295 in the Casitone yeast-extract medium revealed the formation of significant amounts of succinic acid. No additional products were found.

Table 5. *Analysis of glucose (1 %) fermentations by specified strains of Actinomyces spp. and Lactobacillus bifidus in basal medium with Casitone and 1.0 % (w/v) glucose*

	<i>Actinomyces</i> spp. Strain					<i>L. bifidus</i> Strain		
	277	279	282	295	296	307	308	309
	$\mu$ mole used or formed per ml.							
Glucose fermented	25.2	24.9	24.3	26.6	25.4	32.9	49.2	47.6
Carbon dioxide utilized	4.5	8.9	4.7	8.7	10.8	11.2	4.8	0.8
Products								
Formic acid	6.0	7.9	5.8	7.8	11.5	10.4	5.8	7.5
Acetic acid	3.4	3.2	5.4	6.3	12.0	54.8	66.7	70.4
Lactic acid	44.3	42.1	38.2	37.1	24.5	22.1	46.3	38.9
Succinic acid	3.0	4.7	6.1	8.6	12.8	Trace	3.6	1.2
Percentage carbon recovered	101.3	100.7	103.4	98.7	98.1	89.4	97.3	97.4
Redox value	1.00	0.77	1.26	0.94	1.12	0.46	0.98	5.40
Percentage glucose fermented	45.4	44.8	43.8	47.9	45.8	59.2	88.6	85.7

With the change in fermentation noted in the Casitone yeast-extract medium, the necessity for accurate redox balances was emphasized. It had been observed that cultures grown in volumetric flasks from which oxygen was removed by gassing with 95 % N<sub>2</sub> + 5 % CO<sub>2</sub> had long lag periods, gave small amounts of growth and often required re-inoculation to obtain growth. Occasionally similar results were obtained with these flasks using a pyrogallol + Na<sub>2</sub>CO<sub>3</sub> seal. However, cultures in the 25 × 200 mm. test-tubes sealed with pyrogallol + Na<sub>2</sub>CO<sub>3</sub> seals (12 to 15 drops each) invariably gave good growth. These results, together with the fact that carbon dioxide was known to be required by all strains for anaerobic growth, suggested that carbon dioxide was a limiting factor, and that net fixation of carbon dioxide was occurring. Consequently, the fermentation flasks illustrated in Fig. 1 were used with the Van Slyke blood gas apparatus to determine the initial and final carbon dioxide content of the medium. The results presented in Table 5 show that there is a significant net carbon dioxide fixation by *Actinomyces* strains 279, 295 and 296, and probably lesser carbon dioxide fixation by strains 277 and 282, and suggest that carbon dioxide may not be a product of glucose fermentation as indicated by the results in Table 3.



The results in Table 5 show that, in general, the products formed by strains of *Actinomyces* and *Lactobacillus bifidus* on the glucose Casitone medium are identical but differ quite markedly in the relative amounts of acetic acid formed. In these fermentations, the acetic acid formed by actinomycetes accounted for 4.3–15.8 % of the glucose fermented, whereas that formed by the lactobacilli was from 45.2 to 54.5 % of the glucose utilized. Furthermore, of the total glucose added to the medium, the actinomycetes fermented from 43.8 to 47.9 %, whereas the lactobacilli utilized from 59.2 to 88.6 %. All cultures showed an apparent fixation of gaseous carbon dioxide, although in all probability the value given for strain 309 is in error. Strain 307 of *L. bifidus* formed but a trace of succinic acid. Similarly, in other fermentations done with *L. bifidus*, strain 308, succinate was not found.

Negroni & Fischer (1944) and Norris *et al.* (1950) have shown that lactobacilli cultures isolated from human sources form L (+) lactic acid. The type of lactic acid formed by actinomycetes has not been reported. Cultures of strains 277, 279, 282 and 295 were harvested and the lactic acid formed from glucose was isolated as the zinc salt. 4% solutions (w/v) of the anhydrous salts gave specific rotations of  $-5.35$  to  $-8.10$  (specific rotation of a 4 % solution of active zinc lactate was reported by Pederson *et al.* (1926) to be  $-8.25$ ). The 4 % (w/v) solutions of zinc lactate tested were found to be contaminated with a maximum of 0.7 % (w/v) succinic acid. The rotations reported are based on lactic acid concentrations determined by the method of Barker & Summerson (1941). It would therefore appear that L (+) lactic acid is formed by these strains of *Actinomyces* spp. but small amounts of D (–) lactic acid may also be formed.

#### DISCUSSION

It was indicated above that strains representative of *Actinomyces bovis* as described by Erikson (1940) and Thompson (1950) were not included in the present study. Initially two cultures typical of the description of *A. bovis* (Erikson, 1940; Thompson, 1950) were tested for growth on the basal medium used in these experiments (Howell & Pine, 1956). Of these, one strain (284) gave satisfactory growth on this medium but unfortunately this strain was lost during the present investigation. The second strain (278) gave only a limited amount of growth on this medium and therefore was not used in the present study. Attempts were made to obtain other cultures similar to the above two bovine isolates. However, all such cultures available for study were found to be identical, in certain major characteristics (indole,  $H_2S$ , and catalase production; action on milk and gelatin) with *Corynebacterium acnes* as described by Beerens (1953). It was therefore considered unwise to include these cultures in the present study.

Certain strains of *Actinomyces* spp. and *Lactobacillus bifidus* have been described previously as anaerobes or microaerophiles (Erikson, 1940; Gyllenberg, 1955); as requiring or being stimulated by carbon dioxide (Erikson, 1940; Rosebury, Epps & Clark, 1944; Norris *et al.* 1950); of being capable of aerobic growth (Thompson & Lovestadt, 1951; Veltre, Shorb & Pelczar, 1953);

of being able to grow in the presence of small amounts of oxygen (Rosebury *et al.* 1944; Norris *et al.* 1950); of being able to adapt to aerobic conditions (Ludwig & Sullivan, 1952); or, in the case of *L. bifidus*, of being able to adapt to growth in air with the formation of 'straight' rod forms (Gyllenberg, 1955; Hayward, Hale & Bisset, 1955). Erikson & Porteous (1955) have also reported the conversion of a single 'rough' strain of *A. israelii* to a 'smooth' type more tolerant of oxygen. They found, however, that this apparent conversion was due to the presence in cultures of this strain of a facultatively anaerobic staphylococcus which affected both colony morphology and oxygen tolerance of the host strain.

The results reported here show quite clearly that some strains of actinomycetes may be facultative anaerobes, whereas others are microaerophils to obligate anaerobes. Carbon dioxide may or may not be required for growth in air, but is apparently required by all strains of actinomycetes for anaerobic growth under the conditions described above. Of the strains of *Lactobacillus bifidus* tested all required carbon dioxide for anaerobic growth. Only one of the three strains tested grew in air; the growth which was obtained was very limited in amount and carbon dioxide was required. Therefore, it would appear that these strains of *L. bifidus* are essentially microaerophils or anaerobes.

The value of the comparative rates of growth of *Actinomyces* spp. and *Lactobacillus bifidus* on glucose, maltose and lactose is questionable. It would appear that some *Actinomyces* strains grow slowly on lactose, must adapt to its utilization, or lack the ability of utilizing the sugar as efficiently as they do glucose. However, other strains appear to utilize lactose as efficiently as they do glucose or maltose (maltose was utilized as well as glucose by all *Actinomyces* strains tested). Therefore, the use of these sugars for the differentiation of *L. bifidus* from the genus *Actinomyces* would seem to have but limited value, would require exact determination of rates of growth, total cell yields, etc. Nevertheless, considering the results of Rose *et al.* (1953), György, Norris & Rose (1954), Huhtanen (1955), and the results reported above, it would appear that lactose is the preferred sugar for growth of *L. bifidus*, whereas glucose or maltose is the preferred sugar for *Actinomyces* spp.

In most instances, results obtained with *Actinomyces* show that lactic acid was the primary product of glucose fermentation regardless of the medium or conditions employed. In one case reported above (strain 296, Table 5) lactic acid accounted for only 43 % of the glucose fermented; succinic, acetic, and formic acids accounted for the remaining carbon. Depending upon the strain and the medium used, other products were formed but in an amount seldom accounting for more than 20 % of the glucose fermented.

Results obtained with the three strains of *Lactobacillus bifidus* show that lactic and acetic acids in approximately equal amounts (based on the glucose fermented) are the primary products of glucose fermentation, although trace amounts (4-7 % of the glucose fermented) of formic and succinic acids may also be formed. The formation of lactic and acetic acids as the major products of glucose fermentation by human lactobacilli has been reported previously by Orla-Jensen (1943), Negroni & Fischer (1944), and by Norris *et al.* (1950).

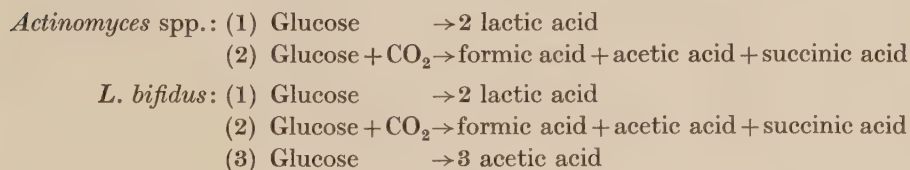
The identity of the compound identified as ethanol in the *Actinomyces* fermentations is questionable since the basal medium contained a small amount of ethanol as a solvent for thioctic acid. The values reported were obtained as small differences between the zero time and final determinations. Acetoin production varied with the medium used, and in general, the greater the concentration of yeast extract or Casitone, the greater the acetoin formation; no acetoin was found to be produced in the glucose casein hydrolysate medium. Acetoin was never formed in amounts sufficiently great to affect the quantitative aspect of the fermentation balances. No other products besides formic, acetic and succinic acids were found in any of the fermentations.

The relative proportion and amounts of the products formed by actinomycetes apparently may be influenced somewhat by the presence of crude organic substances in the medium. Erikson & Porteous (1953) found that on a heart broth casein digest medium, *Actinomyces* strains produced lactic acid equivalent to only 30–60 % of the glucose utilized; other products were not reported.

Changes in the character of fermentations due to changes in the medium or methods of handling the cultures have been described by Pappenheimer & Shaskan (1944), Waring & Werkman (1944), Bard & Gunsalus (1950), and Hodges, Coolidge & Harrison (1951).

Increased carbon dioxide concentrations have previously been shown to be beneficial to the growth of *Actinomyces* strains (Erikson, 1940; Rosebury *et al.* 1944). That strains of *Lactobacillus bifidus* require carbon dioxide for anaerobic growth on solid media has been reported by Norris *et al.* (1950). The fermentative data presented in Table 5 show a net carbon dioxide fixation by both groups of organisms. However, in some cases the values for carbon dioxide fixed as shown in Table 5 are within or closely approaching experimental error. A net fixation in these instances is only suggestive.

If one uses the fermentation data of *Actinomyces* strains 282 or 296, and *Lactobacillus bifidus*, strain 308, as given in Table 5, recognizing the limitations of the data, over-all reactions based on the stoichiometry for glucose utilization for these organisms would approximate the following equations:



The apparent absence of reaction 3 in *Actinomyces* spp. suggests a major metabolic difference between *Actinomyces* spp. and *Lactobacillus bifidus*.

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## The Action of Antibiotics on Indole Synthesis by Cell Suspensions of *Escherichia coli*

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**SUMMARY:** Indole synthesis from glucose, ammonium chloride and serine by washed suspensions of *Escherichia coli* 7-4 was inhibited by bacteriostatic concentrations of chloramphenicol and the tetracyclines (Aureomycin, Terramycin, and Achromycin). Indole synthesis was also inhibited by streptomycin but only by concentrations higher than those necessary for bacteriostasis. The inhibition by chloramphenicol and the tetracyclines does not appear to be due to inhibition of adaptive enzyme formation because these antibiotics still inhibited indole synthesis when added to suspensions which were forming indole at maximum rate. Streptomycin, in the same type of test, seemed to inhibit some adaptive process.

Indole synthesis by suspensions of antibiotic-resistant variants of *Escherichia coli* 7-4 was not inhibited by concentrations of antibiotics which inhibited synthesis by the parent strain. Suspensions of organisms resistant to high concentrations of streptomycin formed at least the same amount of indole in the presence of any concentration of streptomycin tested, as they did in its absence. Streptomycin often stimulated indole synthesis by such organisms. Suspensions of organisms which required streptomycin for growth, after growth in suboptimal concentrations of streptomycin, did not produce appreciable amounts of indole. The addition of streptomycin restored the activity in organisms grown in a liquid defined medium but did not do so with organisms grown on heart infusion agar. Streptomycin-resistant and streptomycin-dependent strains resembled the parent strain in that they utilized malate + pyruvate, or oxalacetate, for indole formation in place of glucose. Strains resistant to various concentrations of chloramphenicol, Aureomycin and Terramycin showed a correlation between the amounts of antibiotic required to inhibit indole synthesis and to inhibit growth in a glucose + tryptophan + inorganic salts medium. The results indicate that some of the antibiotics interfere, either directly or indirectly, with indole synthesis.

Various workers have suggested that some antibiotics may interfere with the biosynthesis of the aromatic amino acids, the evidence being based on the ability of certain aromatic compounds to antagonize the inhibitory action of the antibiotics. Thus it has been suggested that chloramphenicol may interfere with the conversion of anthranilic acid to indole in *Salmonella typhi* (Truhaut, Lambin & Boyer, 1951) and in *Escherichia coli* (Bergmann & Sicher, 1952). Also Foster & Pittillo (1953) found, again with *E. coli*, that tryptophan did not reverse chloramphenicol action although tyrosine and phenylalanine did. Bergmann, Sicher & Volcani (1954), also on the basis of drug reversal tests with *E. coli*, suggested that Aureomycin and Terramycin interfere with the conversion of anthranilic acid to indole. The formation of aromatic compounds from simple precursors (glucose and ammonium ions) by washed suspensions of *E. coli* mutants has recently been described (Gibson,

Jones & Teltscher, 1955*a*, 1956). These experiments have provided a system in which to test the effects of antibiotics on aromatic synthesis more directly. The formation of indole by substrains of *E. coli* 7-4 made resistant to streptomycin, chloramphenicol and the tetracyclines has been compared with that by the antibiotic-sensitive strain. Part of this work has already been briefly reported (Gibson, Jones & Teltscher, 1955*b*).

## METHODS

*Preparation and use of suspensions of Escherichia coli.* The methods used for the study of formation of indole by washed suspensions of *Escherichia coli* were described in detail by Gibson *et al.* (1956). Briefly, the organisms were harvested from 16 hr. cultures on heart infusion agar plates, washed twice in saline, and resuspended in 0.2 M-phosphate buffer (pH 7.7) to give the equivalent of about 1.5 mg. dry wt. organisms/ml., and used at once. The suspensions were then added to an equal volume of substrates and incubated at 37°. Indole formation was measured colorimetrically by using Ehrlich's reagent.

The organisms used were either *Escherichia coli* strain 7-4 (referred to as the parent strain), a mutant requiring tryptophan for growth, or antibiotic-resistant strains derived from the parent strain.

*Basal synthesis system.* Unless otherwise stated, the effects of the antibiotics were measured on the formation of indole in the following medium: glucose (0.1 M);  $\text{NH}_4\text{Cl}$  (0.005 M); DL-serine (0.01 M) incubated at 37° in 0.1 M-phosphate buffer pH 7.7. The tests were carried out in a final volume of 5 ml. in  $6 \times \frac{3}{4}$  in. tubes.

*Antibiotics.* The antibiotics used were Glaxo penicillin and streptomycin sulphate, Parke-Davis chloramphenicol, the hydrochlorides of Pfizer Terramycin (oxytetracycline), Lederle Aureomycin (chlortetracycline) and Achromycin (tetracycline). The concentrations of streptomycin used are expressed in terms of free base. In experiments in which bacteriostatic tests were carried out in parallel with tests on indole formation the following procedures were adopted. Streptomycin was added to the tubes of the bacteriostatic test before autoclaving. Chloramphenicol was either autoclaved with the medium or a solution of 1000  $\mu\text{g.}/\text{ml.}$  was pasteurized at 70° for 10 min. and dilutions in sterile distilled water added aseptically to the tubes. The tetracyclines were prepared immediately before use and were pasteurized as above at 1 mg./ml. During earlier experiments, including some of those described by Gibson *et al.* (1955*b*), solutions of Aureomycin and Terramycin were prepared from capsules intended for oral administration. However, gifts of pure tetracyclines made it possible to use these for most of the work.

*Bacteriostatic tests.* These were carried out in the chemically defined medium described by Davis & Mingioli (1950) with the addition of  $10^{-4}\text{M}$ -DL-tryptophan necessary for the growth of *Escherichia coli* 7-4. The glucose and tryptophan were added to the medium before autoclaving. Double-strength medium was tubed in 2.5 ml. volumes in  $6 \times \frac{3}{4}$  in. acid-washed tubes; water and drug were added and the tubes autoclaved at 10 lb./sq.in. for 10 min. Any further



additions were made aseptically. The inoculum was one drop (about 0.03 ml.) of a light suspension (about  $2 \times 10^6$ /ml.) of the organisms to be used. After incubation for 24 hr. at 37° the turbidity was measured with the E.E.L. portable colorimeter with  $\frac{1}{4}$  in. diam. tubes and a 626 filter. The amount of growth was expressed in terms of scale reading; these two were directly proportional over the range used and a turbidity reading of 10 is equivalent to 0.3 mg. dry wt./ml.

*Drug-resistant organisms.* Drug-resistant substrains of *Escherichia coli* 7-4 were obtained either by direct selection from large populations or by serial subculture in increasing concentrations of the given antibiotic. Strains highly resistant to, or dependent on, streptomycin were isolated by washing off the growth from 24 hr. cultures on two heart infusion agar plates with the minimum amount of sterile distilled water and spreading the suspension over the surface of a heart infusion agar plate containing 400 µg. streptomycin/ml. Any colonies which appeared during incubation at 37° for up to 4 days were subcultured and tested for resistance and dependence. Organisms showing a three- to fivefold increase in resistance were obtained by serial subculture of the parent strain in subinhibitory concentrations of streptomycin (Gibson & Gibson, 1951).

Strains resistant to chloramphenicol and the tetracyclines were obtained from a series of bacteriostatic tests in defined medium, the inoculum for each new set of tubes being taken from the highest concentration of antibiotic which allowed visible growth in 24 hr. Strains were sometimes obtained which had lost the ability to form indole. The reason for this is not known; such strains were discarded and a new series started from the parent strain.

Antibiotic-resistant strains were maintained on heart infusion agar slopes containing the appropriate antibiotic or, for strains resistant to high concentrations of streptomycin, on plain heart infusion agar slopes.

*Preparation of suspensions of streptomycin-dependent organisms grown with minimal streptomycin.* Streptomycin-deficient organisms were prepared by growing strains requiring streptomycin in suboptimal concentrations of streptomycin either on heart infusion agar plates or in a liquid synthetic medium.

Five ml. of the defined medium containing 20 µg. of streptomycin/ml. was inoculated with the dependent strain and incubated for 24 hr. at 37°. This culture (0.2 ml.) was used as the inoculum for plates of heart infusion agar containing streptomycin which were then incubated for 20 hr. at 37°. The organisms were then harvested and washed in the usual way before use. Alternatively, Roux bottles containing 150 ml. of the defined medium + streptomycin were inoculated with 1 ml. of culture and incubated as above. Organisms were then spun down, washed twice with saline and resuspended in buffer.

*Nomenclature of resistant strains.* Strains resistant to, but not dependent on, streptomycin are referred to as SR, those resistant to chloramphenicol as CR and to Terramycin as TR. Strains which required streptomycin for growth are referred to as SD.



## RESULTS

*Antibiotics inhibiting indole synthesis by the parent strain*

The effects of antibiotics on indole formation are shown in Table 1. Penicillin when added at five times the bacteriostatic concentration (100 units/ml.) had no inhibitory effect and even at 1000 units/ml. had a relatively small effect.

Table 1. *The effects of antibiotics on indole synthesis and growth of the parent strain (Escherichia coli 7-4)*

For indole synthesis washed organisms were incubated with basal synthesis system (glucose, 0.1 M;  $\text{NH}_4\text{Cl}$ , 0.005 M; DL-serine, 0.01 M; in 0.1 M-phosphate buffer pH 7.7) for 4½ hr. Bacteriostatic tests in minimal medium + tryptophan (see Methods). In this and all succeeding experiments shown, the amount of organisms used for indole synthesis was within the range 0.65–0.80 mg. dry wt./ml. and the temperature of incubation was 37°.

Antibiotic	Bacteriostatic tests		Indole synthesis	
	Growth in	No growth in	Conc. antibiotic added	Indole formed (mμmole/mg.)
None	—	—	—	276
Terramycin (μg./ml.)	0.25	0.5	1	90
Aureomycin (μg./ml.)	0.25	0.5	1	73
Achromycin (μg./ml.)	0.25	0.5	1	78
Chloramphenicol (μg./ml.)	2	3	5	73
Streptomycin (μg./ml.)	2	3	10	282
			150	146
Penicillin (units/ml.)	10	20	100	266
			1000	255

Chloramphenicol and the tetracyclines were found to inhibit indole synthesis markedly, even at about bacteriostatic concentrations.

Streptomycin at bacteriostatic concentration (3 μg./ml.) did not inhibit the formation of indole but did so markedly at higher concentrations (150 μg./ml.).

The results of more detailed experiments are presented below.

*Chloramphenicol.* There was a marked correlation between the concentrations inhibiting growth and those depressing indole formation. As can be seen from Fig. 1 a detectable inhibition of indole synthesis took place even at concentrations of the antibiotic below that required to inhibit growth completely.

*Tetracyclines.* The three tetracyclines all caused the same type of response as found with chloramphenicol. As an example, the results of an experiment with Achromycin are illustrated in Fig. 2. Once again indole synthesis was inhibited to some extent by antibiotic concentrations lower than those required for growth inhibition.

*Streptomycin.* The concentration of this antibiotic which completely inhibited growth had no appreciable effect on indole synthesis, though it was progressively inhibited at concentrations between 10 and 120 μg./ml. (Fig. 3). Above 120 μg./ml. there was only a slight increase in inhibition.

*The effect of time of addition of antibiotics.* The rate of indole synthesis increases during the first 2 hr. until a maximum rate is reached (Gibson *et al.*

1956). The effect of adding antibiotics to cells producing indole at the maximum rate is shown in Fig. 4. Once again the action of streptomycin was found to differ distinctly from that of chloramphenicol and the tetracyclines. On the addition of either of the latter antibiotics indole synthesis was rapidly inhibited.

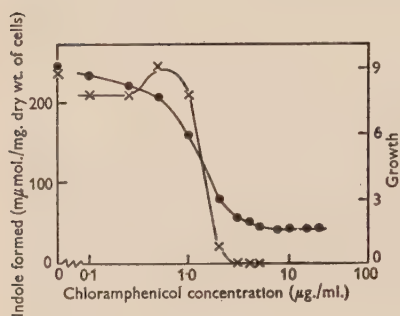


Fig. 1

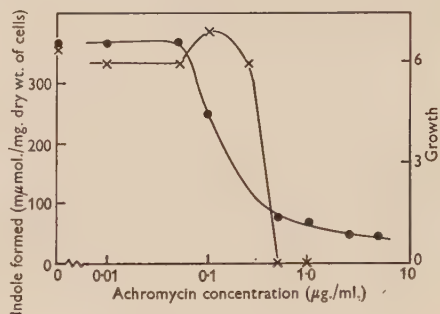


Fig. 2

Fig. 1. Effect of chloramphenicol on indole synthesis and growth. — × — × —, growth (see Methods); —●—●—, indole synthesis after 4 hr. incubation.

Fig. 2. Effect of Achromycin on indole synthesis and growth. — × — × —, growth; —●—●—, indole synthesis after 5 hr. incubation.

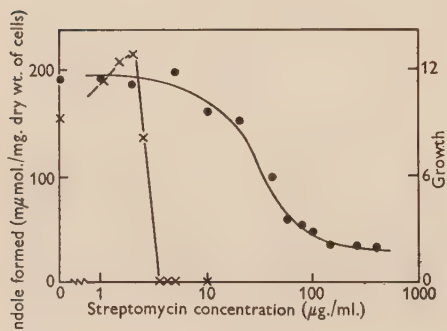


Fig. 3

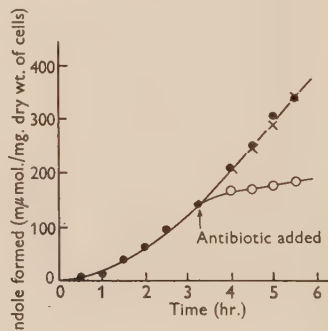


Fig. 4

Fig. 3. Effect of streptomycin on indole synthesis and growth. — × — × —, growth; —●—●—, indole synthesis after 4 hr. incubation.

Fig. 4. The effect of antibiotics on cells forming indole at maximum rate. Organisms incubated initially in 80 ml. final volume. Immediately before addition of drugs, organisms and substrates were divided into 10 ml. lots; 0.1 ml. of each of the drugs added to give the final concentration shown below. —●—●—, no addition; — × — × —, 150 µg. streptomycin/ml.; —○—○—, 1 µg. Terramycin/ml. or 1 µg. Aureomycin/ml. or 5 µg. chloramphenicol/ml.

On the other hand, the addition of streptomycin at a concentration (150 µg./ml.) which would inhibit synthesis if added initially, did not affect the rate of synthesis. In some experiments in which the synthesis was observed for a longer time (e.g. 5 hr.) after the addition of streptomycin, some inhibition was observed.

*Streptomycin-resistant cells*

Strains of *Escherichia coli* resistant to streptomycin fall into three groups (Umbreit, 1952), which are considered in turn.

*A. Highly resistant organisms.* Although with the parent strain streptomycin in concentrations greater than 10  $\mu\text{g./ml.}$  inhibited indole formation, indole synthesis by the highly resistant strains was never inhibited by any concentration of streptomycin tested, up to 2000  $\mu\text{g./ml.}$  On the contrary, indole synthesis was frequently stimulated (Fig. 5). Resistant strains usually, but not always,

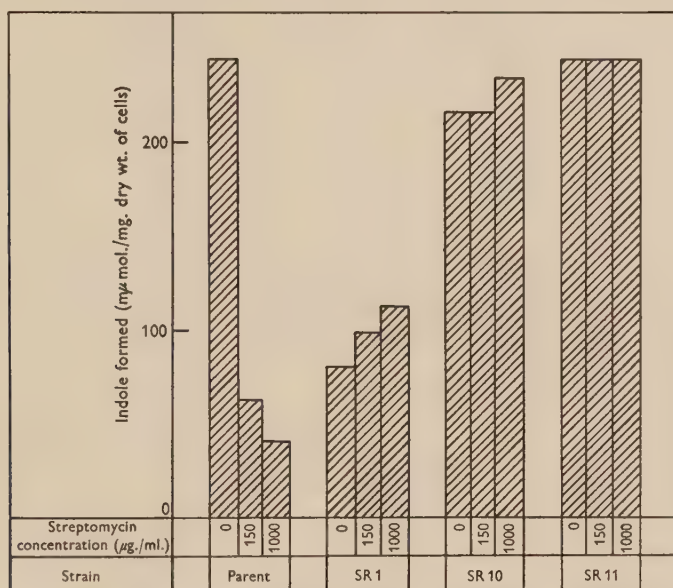


Fig. 5. Effect of streptomycin on indole synthesis by the parent strain and strains highly resistant to streptomycin. Cells incubated in basal synthesis system for  $4\frac{3}{4}$  hr., together with streptomycin as shown.

formed less indole than the parent strain. This loss of synthesizing power could be largely overcome by the addition of magnesium ions to the basal synthesis system (Table 2).

*B. Organisms slightly resistant to streptomycin.* An extensive investigation of indole synthesis by these organisms has not been carried out but the effect on it of streptomycin did not appear to differ significantly from that on indole synthesis by the parent strain.

*C. Streptomycin-dependent organisms.* Suspensions of a strain which required streptomycin for growth, harvested from media containing 500  $\mu\text{g./ml.}$  of streptomycin, formed indole in the presence of all concentrations of streptomycin tested. As with the highly resistant organisms, indole formation was often stimulated by streptomycin (Table 3).

However, SD organisms grown on the chemically defined medium containing suboptimal concentrations of streptomycin, failed to form appreciable

amounts of indole in the absence of streptomycin. Similar organisms from heart infusion agar formed no appreciable indole even when streptomycin was added to the synthesis system (Table 3, Fig. 6). At the conclusion of experiments

Table 2. *Streptomycin-resistant organisms: stimulation of indole synthesis by magnesium*

Suspensions were incubated with substrates for 4 hr.

Streptomycin-resistant strain	Indole formed (m $\mu$ mole/mg.)	
	Basal synthesis system	Basal synthesis system + MgSO <sub>4</sub> (10 <sup>-4</sup> M)
SR 1	81	181
SR 2	195	278
SR 3	113	286
SR 4	127	233
SR 5	108	283
SR 6	123	280

Table 3. *Effect of streptomycin on indole synthesis by a streptomycin-dependent strain*

Cells were incubated in basal synthesis system + 10<sup>-4</sup>M-MgSO<sub>4</sub> for 5 hr.

Organisms grown on		Indole synthesis (m $\mu$ mole/mg.)	
Medium	Streptomycin ( $\mu$ g./ml.)	No added streptomycin	Streptomycin (1000 $\mu$ g./ml.)
Liquid defined	20	22	166
	500	205	206
Heart infusion agar	20	44	50
	500	338	377

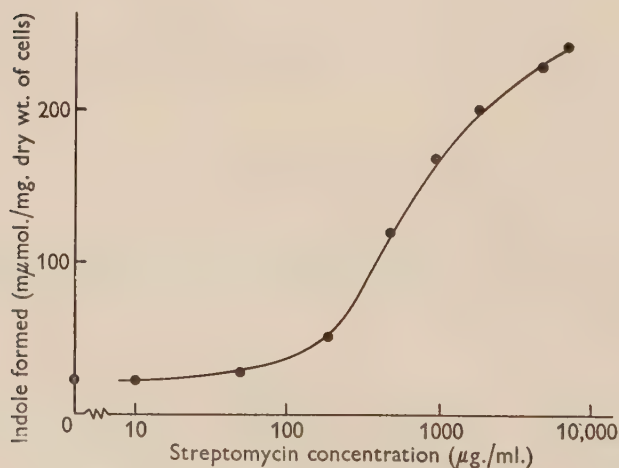


Fig. 6. Effect of streptomycin on indole synthesis by a streptomycin-dependent strain. Organisms harvested from defined liquid medium containing limiting streptomycin (20  $\mu$ g./ml.). Basal indole synthesis system + MgSO<sub>4</sub> (10<sup>-4</sup>M) and streptomycin as shown. Incubation for 5 hr.



with SD organisms the suspensions were plated to find out whether they still required streptomycin. While a few colonies did sometimes appear on medium containing no streptomycin, the bulk of the organisms still required streptomycin. There was no increase in the turbidity of the suspensions during the experiments.

*The utilization of oxalacetate and pyruvate plus malate by resistant and dependent strains.* The glucose in the basal synthesis system can be replaced by pyruvate plus malate but not by either alone (Teltscher & Gibson, unpublished observations), or by oxalacetate. In view of the observations of Smith, Oginsky & Umbreit (1949) concerning alterations in the metabolism of these compounds in streptomycin-resistant and -dependent organisms, it was of interest to compare their utilization for indole synthesis by the different strains. It was found (Table 4) that pyruvate plus malate, or oxalacetate, were utilized for indole formation by both the resistant and dependent strains tested.

Table 4. *Utilization of malate plus pyruvate, and oxalacetate, by resistant and dependent strains*

Organisms in 0.2M-phosphate buffer pH 7.7 were incubated (4 hr. Expt. 1, 5 hr. Expt. 2) with substrates shown +MgSO<sub>4</sub> (10<sup>-4</sup>M). Substrate concentrations; glucose, malate, pyruvate and oxalacetate 0.1M, NH<sub>4</sub>Cl 0.005M. Streptomycin-dependent cells grown on heart infusion agar containing 500 µg./ml. streptomycin.

Organism	Indole formed (mµmole/mg.) with the following substrates			
	Expt. 1		Expt. 2	
	Glucose NH <sub>4</sub> Cl	Malate pyruvate NH <sub>4</sub> Cl	Glucose NH <sub>4</sub> Cl	Oxal- acetate NH <sub>4</sub> Cl
<i>Escherichia coli</i> 7-4 (parent strain)	256	137	267	161
SR 5 (streptomycin-resistant)	212	228	200	190
SD 1 (streptomycin-dependent)	156	214	158	183

#### *Strains resistant to chloramphenicol*

The concentration of chloramphenicol needed to inhibit indole synthesis by suspensions of several resistant strains capable of growing in a concentration of about 100 µg. chloramphenicol/ml. was about the same as that needed to inhibit growth in the chemically defined medium. The result of a typical experiment is shown in Fig. 7.

#### *Strains resistant to the tetracyclines*

The effects of these antibiotics on indole synthesis and growth showed the same pattern as described above for chloramphenicol. The results shown in Fig. 8 indicate that even a relatively slight increase in resistance is paralleled by a similar effect on indole synthesis. A further example with cells resistant to a much higher concentration of Terramycin is shown below (Fig. 9). Similar results were obtained with strains resistant to Aureomycin.

## Cross-resistance

Organisms resistant to chloramphenicol, to Terramycin and to Aureomycin were tested to see if cross-resistance would be shown either in growth inhibitions or with regard to indole synthesis. No cross-resistance in either respect was demonstrated by organisms resistant to chloramphenicol. Organisms resistant

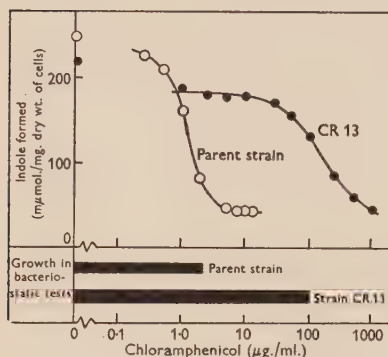


Fig. 7

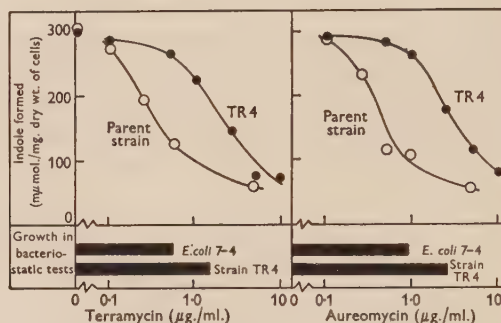


Fig. 8

Fig. 7. Effect of chloramphenicol on indole synthesis and growth by the parent strain and a strain (CR13) resistant to chloramphenicol. Indole synthesis: suspensions incubated in basal synthesis system with concentrations of chloramphenicol as shown for 4 hr. Bacteriostatic tests: 24 hr. in defined medium (see Text). Horizontal bars represent growth in the concentration of chloramphenicol shown.

Fig. 8. The effects of Terramycin and Aureomycin on the parent strain and a Terramycin-resistant strain (TR4). Data as for Fig. 4 except in indole synthesis: parent strain 4 hr. incubation; strain TR4 5 hr. incubation.

Table 5. Cross-resistance in bacteriostatic tests: increased resistance to *Achromycin*, *Aureomycin* and *chloramphenicol* of strain (TR13) resistant to *Terramycin*

Antibiotic	Highest conc. (μg./ml.) allowing growth	
	Parent strain	Strain TR 13
Achromycin	0.25	20
Terramycin	0.25	20
Aureomycin	0.5	20
Chloramphenicol	2	50

to Terramycin or Aureomycin were not only resistant to the other tetracyclines but also to chloramphenicol (Table 5 and Figs. 8 and 9). Fig. 8 shows that this held good with organisms only slightly resistant to Terramycin. The effects of antibiotics on indole synthesis by organisms more highly resistant to Terramycin (Table 5) are shown in Fig. 9. Indole synthesis by these resistant cells was much less inhibited by the other tetracyclines and by chloramphenicol than synthesis by the parent strain.

## DISCUSSION

Studies on the action of the tetracyclines and chloramphenicol on biochemical reactions using cell suspensions or extracts have usually shown either no inhibition, or inhibition at concentrations higher than bacteriostatic. However, exceptions have been shown (Gale & Folkes, 1953; Saz & Marmur, 1953; Wisseman, Smadel, Hahn & Hopps, 1954; Hahn & Wisseman, 1951). Chloramphenicol, Aureomycin and Terramycin were found by Hahn & Wisseman (1951) to inhibit adaptive enzyme formation in suspensions of *Escherichia coli*. In the present experiments these antibiotics, when added at bacteriostatic

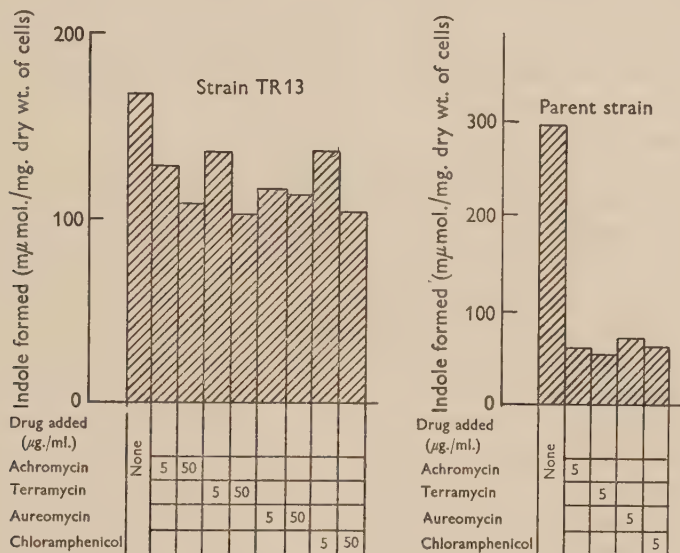


Fig. 9. Effect of antibiotics on indole synthesis by the parent strain and a strain (TR13) resistant to Terramycin. Details as for Fig. 3. Incubation for 5 hr.

concentrations, inhibit the synthesis of indole from glucose, ammonium chloride and serine by suspensions of *E. coli*. Although the rate of indole synthesis is typical of an adaptive process (Gibson *et al.* 1956) it does not seem that either chloramphenicol or the tetracyclines are interfering with adaptive enzyme formation alone, in this case, since the addition of antibiotic while indole synthesis is proceeding at maximum rate still causes inhibition.

That the tetracyclines and chloramphenicol may inhibit the same or related reactions is supported by the observation that cross-resistance can develop between these antibiotics (Herrel, Heilman & Wellman, 1950; Pansy, Khan, Pagano & Donovick, 1950).

The questions as to which stage or stages in aromatic synthesis are inhibited by these antibiotics, and whether their action is directly on reactions in the synthetic pathway, have yet to be answered. The fact that not only tryptophan, but several of the other aromatic amino acids, have some action in



antagonizing the action of chloramphenicol (Bergmann *et al.* 1954; Foster & Pittillo, 1953; Woolley, 1950; Truhaut *et al.* 1951) suggests that this drug may well interfere with some reaction common to the synthesis of these amino acids. Preliminary experiments with washed suspensions of a strain of *Escherichia coli* which forms anthranilic acid indicate that anthranilic acid synthesis is also inhibited by Terramycin and chloramphenicol.

Streptomycin at bacteriostatic concentrations does not inhibit indole synthesis. Therefore it seems likely that its effect on aromatic synthesis is not the primary cause of bacteriostasis. However, the process which is inhibited by the higher concentrations is presumably of importance during the development of resistance to high levels of streptomycin. Streptomycin appears to inhibit some process which is adaptive since the addition of the antibiotic, once the cells are forming indole at their maximum rate, no longer has any immediate effect. Whether the process inhibited is actually that of adaptive enzyme formation is still an open question.

Comparison of the effects of the antibiotics on indole synthesis by the parent and drug-resistant strains shows that indole synthesis by the resistant strains is much less susceptible to the action of the corresponding antibiotic.

Although it seems likely that the reactions inhibited by low concentrations of streptomycin may not be directly concerned with aromatic synthesis, for cells to become highly resistant the reactions (reflected here by indole synthesis) inhibited by higher concentrations of streptomycin must become insusceptible to the drug in some way or replaced by an alternative metabolic pathway.

Oxygen uptake in the presence of pyruvate plus oxalacetate by aged suspensions of *Escherichia coli* was found by Oginsky, Smith & Umbreit (1949) to be inhibited by low concentrations of streptomycin. It was suggested that streptomycin inhibited some metabolic pathway involving a pyruvate-oxalacetate condensation. Strains which were resistant to, or dependent on, streptomycin were found to have lost the ability to oxidize pyruvate plus oxalacetate, and it was suggested that such cells had developed an alternative metabolic pathway (Smith *et al.* 1949). In the present experiments it has been found that oxalacetate, or malate plus pyruvate, will serve as substrates for indole synthesis not only with *E. coli* 7-4, but also with the streptomycin-resistant and dependent substrains. With the fresh organisms used pyruvate would be produced from oxalacetate (Oginsky *et al.* 1949). If an oxalacetate-pyruvate condensation takes place during indole synthesis under the conditions used, the resistant and dependent organisms apparently still retain the ability to carry out the reaction. The results obtained with streptomycin during the present work indicating that it may inhibit some adaptive process are similar to those of Roote & Polglase (1955) who tested the effects of dihydrostreptomycin on the formation of adaptive enzymes by suspensions of *E. coli*. These workers found that the formation of adaptive enzymes for the oxidation of certain carbohydrates was inhibited by 1000  $\mu$ g. dihydrostreptomycin/ml. The same concentration did not significantly inhibit the formation of adaptive enzymes in resistant organisms and stimulated their formation slightly in dependent organisms; those deficient in streptomycin were not tested.



Organisms dependent on streptomycin for growth could apparently be made streptomycin-deficient by growing them in a medium containing suboptimal concentrations of streptomycin. Suspensions of such organisms prepared from a liquid defined medium had largely lost the power to form indole, the activity being restored by the inclusion of streptomycin among the substrates for indole synthesis. Such results, if compared with studies on the function of growth factors, are consistent with the view that in organisms requiring streptomycin for growth the antibiotic may act as a co-enzyme in some vital reaction. It is of interest that indole synthesis by suspensions of streptomycin-resistant organisms was often stimulated by streptomycin. Streptomycin-deficient organisms harvested from heart infusion agar formed very little indole but in this case the activity could not be restored by the addition of streptomycin.

In the case of organisms resistant to chloramphenicol and tetracyclines, the correlation between the minimal bacteriostatic concentration and the amount of antibiotic required to inhibit indole synthesis supports the view that these antibiotics inhibit vital reactions concerned in aromatic synthesis.

Strains of *Escherichia coli* made resistant to either chloramphenicol, Terramycin or Aureomycin have been found to show a cross-resistance to the other two drugs (Herrel *et al.* 1950; Pansy *et al.* 1950). During the present experiments strains resistant to chloramphenicol were not found to show any cross-resistance. However, strains cultivated in the presence of either Aureomycin or Terramycin showed a cross-resistance not only to the other tetracyclines but also to chloramphenicol. This cross-resistance was shown by growing organisms and by indole synthesis in suspensions. The present evidence does not provide any answer as to the way in which the resistant organisms are able to carry out biochemical processes in the presence of concentrations of antibiotics which inhibit the same processes in sensitive organisms. The study of aromatic synthesis provides an experimental approach in this problem. The results presented suggest that several antibiotics interfere with reactions associated with aromatic synthesis and that examination of these reactions may be of use in studying their mode of action.

We wish to thank Mr G. Morrison for technical assistance and Dr Margaret Gibson for her advice. We are grateful to Professor S. D. Rubbo for his encouragement of this work and to the National Health and Medical Research Council for a grant.

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## *Pichia vanriji* n.sp., Isolated from Soil

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**SUMMARY:** A new *Pichia* species has been isolated from soil. Its characteristic features are its large cells, the abundant formation of pseudomycelium, an early appearance of a pellicle, and the formation of four-spored asci. The species is non-fermentative and assimilates only glucose, galactose, sucrose and maltose.

In the course of a study of the yeast flora associated with surface soil two strains of an apparently undescribed *Pichia* species were isolated.

### METHODS AND RESULTS

The following description is based on the standard procedures described by Lodder & Kreger-van Rij (1952).

#### *Description*

*Growth in malt extract.* After 3 days at 25° the cells are round (2.5-8.8)  $\mu$ . or oval (2.5-7.5)  $\times$  (3.1-11.3)  $\mu$ ., single or in pairs. A dull, dry, wrinkled, creeping pellicle is formed. After 1 month a sediment is formed as well.

*Growth on malt agar.* After 3 days at 25° the cells are round (3.1-10.0)  $\mu$ . or oval (3.1-8)  $\times$  (3.8-13.1)  $\mu$ ., single or in pairs. Copulating cells and asci are also present. After 1 month at 17° the streak culture is greyish to yellowish white and wrinkled. The margin is fringed with pseudomycelium.

*Slide cultures.* A pseudomycelium of the 'mycocardia' type is abundantly formed.

*Sporulation.* Sporulation was observed on malt agar. A heterogeneous conjugation between a cell and its bud usually precedes ascus formation. The ascospores are round, smooth, and often contain an oil droplet. One to four spores are formed per ascus (Fig. 1).

*Fermentation.* Absent.

*Sugar assimilation.* Glucose, +; galactose, +; sucrose, +; maltose, +; lactose, -.

*Ethanol as sole source of carbon.* Growth occurs as a pellicle.

*Assimilation of potassium nitrate.* Absent.

*Splitting of arbutin.* Positive.

### DISCUSSION

The strains are included in the genus *Pichia* since their vegetative reproduction is characterized in the first instance by the early formation of a dull creeping pellicle and secondly by the abundant production of a pseudomycelium.

Within this genus no species has hitherto been described which assimilates only glucose, galactose, sucrose and maltose. Because of this significant biochemical difference the strains must consequently be regarded as representative of a new species. For this species we propose the name *Pichia vanriji*, in honour of Mrs N. J. W. Kreger-van Rij, mycologist at the Yeast Division of the Centraal Bureau voor Schimmelcultures in Delft.

Morphologically, *Pichia vanriji* is also quite distinct and its close relationship with the other members of the genus, *P. farinosa* (Lindner) Hansen and *P. polymorpha* Klöcker, quite apparent. However, the strains possess an



Fig. 1. *Pichia vanriji*. Ascospores on malt agar after 7 days.  $\times 1400$ .

undeniable tendency towards the formation of round cells which, in its turn, is suggestive of the genus *Debaryomyces* as emended by Lodder & Kreger-van Rij (1952). These authors have, on the other hand, pointed out the close relationship between the genera *Pichia* and *Debaryomyces* and have already remarked on the existence of intermediate forms such as *Debaryomyces vini* Zimmermann which takes a unique intermediate place between the two genera.

A culture of *Pichia vanriji* has been deposited in the Yeast Collection of the Central Bureau voor Schimmelcultures in Delft.

#### LATIN DIAGNOSIS

##### *Pichia vanriji*, sp. nov.

In musto maltato cellulae rotundae (2·5–8·8)  $\mu$ ., aut ovidiae (2·5–7·5)  $\times$  (3·1–11·3)  $\mu$ ., singulae aut binae. Post dies 3 pellicula non-nitida, crispulata, sursum repens formatur. Sedimentum.

In agaro maltato cellulae rotundae (3·1–10)  $\mu$ ., aut ovidiae (3·1–8·1)  $\times$  (3·8–13·1)  $\mu$ ., singulae aut binae. Cellulae conjugatae et asci. Cultura (post unum mensem, 17°) griseola flavalbida, crispulata, non-nitida; margine piloso. Pseudomycelium abundat.

Copulatio cellularum inaequarum plerumque conformationem asci praecedet. Ascosporae rotundae, glabrae, fortasse globulos olei continent. 1–4 in asco.



Fermentatio nulla. In medio minerali cum glucoso, galactoso, saccharo et maltoso crescit. Nitras kalicus no assimilatur. In medio minerali cum alcohole aethylico crescit, pellicula formatur. Arbutinum finditur.

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#### REFERENCE

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## The Adaptive Formation of Levansucrase by a Species of *Corynebacterium*

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**SUMMARY:** Levansucrase in the corynebacterium studied is apparently an adaptive enzyme. Its optimal formation is dependent on the presence of an inducer (sucrose) and an organic nitrogen source. High nitrogen concentrations increase growth rate and sucrose hydrolysis and significantly decrease levansucrase formation. The pH activity curve of the levansucrase studied is asymmetric and the optimal pH value for its activity is 7.0. These findings suggest that this levansucrase differs from other levansucrases so far described.

In a preliminary note (Henis & Aschner, 1954), levan formation by a species of *Corynebacterium* was described. Maximal yield of levan was obtained in a medium composed of 0.1 % (w/v)  $K_2HPO_4$  and 10 % (w/v) sucrose in tap water. Only unwashed organisms grown on ordinary nutrient agar could serve as inoculum since washed organisms lost most of their levan-synthesizing ability. It was found later that by the addition of small amounts of certain organic nitrogen compounds (peptone, casein hydrolysate, glutamic acid) the levan-synthesizing ability of washed organisms could be regained. The purpose of this work is to evaluate some factors affecting levan formation by the organism studied.

### METHODS

**Organism.** The organism used was that isolated and described by us as a species of *Corynebacterium* (Henis & Aschner, 1954).

**Inoculum.** The organism was grown for 48 hr. at 30° on a medium composed of (% w/v): glucose, 2; peptone, 0.5; Marmite, 0.5; agar, 2.5; in distilled water. The pH value was adjusted to 7.7 after sterilization. The organisms were washed 3 times with saline, stored in a refrigerator and used during 7 days; their viability did not change during that period. The number of organisms in the inoculum was determined in a Coleman junior spectrophotometer at 550 m $\mu$ ., using a calibration curve obtained by the plate count method.

**Media.** Bacto-peptone ('Difco' standardized) or glutamic acid in concentrations up to 10 mg./ml. were used as a nitrogen source. The following solutions served as mineral source: (1) boiled and filtered tap water; (2) a solution containing (mg./l.):  $MgSO_4 \cdot 7H_2O$ , 60;  $Na_2CO_3$ , 200;  $CaCl_2$ , 4.5;  $Fe_2(SO_4)$  3, 0.01. The buffer used was Sorensen's phosphate buffer (pH 7.6). Sucrose, in concentrations up to 100 mg./ml. served as a substrate for both the levansucrase and the sucrase systems, and as a part of the carbon and energy sources.

**Sterilization** was at 120° for 15 min.

*Aeration and continuous suspension of the organisms* were obtained by the use of a roller tube (usually used for tissue culture) which rotated 5 rev./hr. Screw-cap test tubes (19 mm. diam.), each containing 7 ml. medium, were put into the rotating instrument in an horizontal position. In this way, the formation of levan gel (Henis & Ascher, 1954) in the media was avoided.

The incubation temperature in all experiments with growing organisms was 30°.

*Growth determinations* were carried out with a Coleman junior spectrophotometer at 550 m $\mu$ . by means of a calibration curve against plate count. The turbidity caused by levan formation during growth was eliminated by a mild acid hydrolysis, which did not change the turbidity caused by the growing organisms. Preliminary determinations of levan were made by measuring the turbidity of the levan and the bacteria before and after a mild hydrolysis and subtracting the final turbidity values from the total turbidity. Levan solutions of known concentrations were used as standard solutions.

*Determinations of levan and reducing sugar* were made by the methods of Somogyi (1945) and Nelson (1944). Levan was prepared for quantitative determination according to the method of Avineri-Shapiro & Hestrin (1945). All values were expressed as mg. glucose/ml. Glucose solutions of known concentrations were used for standardization purposes.

*Determination of protein in cell-free extracts* was made by the method of Mehl (1945), with egg albumin solution as a standard.

*Preparation of enzyme solutions.* Organisms, at a final concentration of  $10^9$  organisms/ml., were added to Roux bottles, each containing 100 ml. medium (10 g. sucrose, 0.03 g. peptone, in M/30 phosphate buffer, pH 7.6, in tap water) and incubated for 18 hr. at 30°. The organisms, together with part of the levan formed, were then collected in a Sharples supercentrifuge and put in a Raytheon 9 Kc sonic vibrator for 4 min. This treatment decreased the viscosity of the levan and enabled the separation of the organisms, which otherwise precipitated with the levan during centrifugation. The unbroken organisms were collected in a MSE high-speed centrifuge at 10,000 r.p.m. for 10 min., washed 3 times with saline and diluted to 1/100 of the original volume of the medium. The organisms were then put again in the sonic vibrator for 40 min. and the cell debris separated in the high-speed centrifuge. The cell-free extract contained 3 mg. protein/ml. Protein was precipitated by saturation with ammonium sulphate, and the precipitate dialysed overnight against distilled water and diluted with physiological saline to a final concentration equivalent to 1.5 mg. protein/ml. The extract was free from levan and reducing sugar as judged by the methods used by us. In a similar way, cell-free extracts from organisms grown under various other conditions were prepared.

*Composition of reaction mixture and determination of enzymic activity.* Reaction mixtures of cell-free extracts contained 0.5 mg./ml. protein, 50 mg./ml. sucrose and M/45 phosphate buffer in distilled water. For pH values higher than 7.8 M/30 KCl + borate + NaOH mixtures were used. In all cases, enzymic activity was determined with 3 ml. volumes of reaction mixture which were incubated in a 37° water bath for 2 hr.

*Paper chromatography* was carried out on Whatman filter-paper no. 1 with *n*-butanol/acetic acid/water mixture (4 : 1 : 5; Partridge, 1948) as a solvent. Benzidine + trichloroacetic acid (Bacon & Edelman, 1951) was used as a colour-developing reagent. Ascending chromatograms were run for 48 hr. at 30°.

## RESULTS

### *Experiments with growing organisms*

*Effect of nitrogen concentration on levan synthesis.* Experiments were carried out in four series; the results are shown in Figs. 1 and 2. The amount of sucrose hydrolysed was calculated by subtracting the glucose values equivalent to the levan formed from the total amount of reducing sugar which appeared in the medium. This calculation was based on the reaction, adapted from Hehre (1955):



when all the fructose part of the sucrose is incorporated into levan. No more than 5% (w/v) of the sucrose was used by the organism as an energy source.

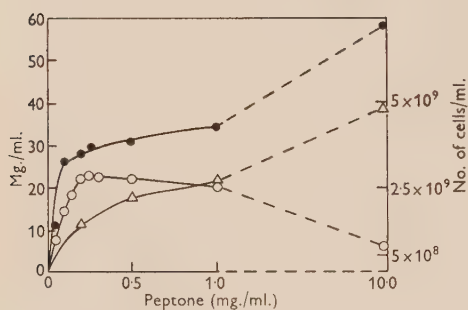


Fig. 1

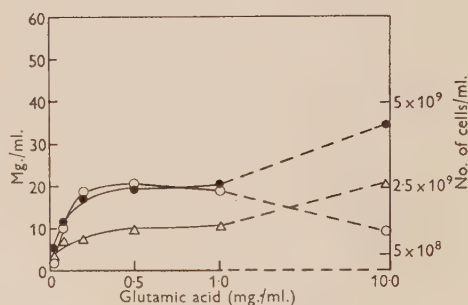


Fig. 2

Fig. 1. Levan production, sucrose hydrolysis and multiplication as a function of peptone concentration. In addition to the nitrogen source, the medium contained 100 mg. sucrose/ml. and M/30 phosphate buffer in tap water. Organisms, at a final concentration of  $5 \times 10^8$  organisms/ml., were added to test tubes containing the medium and incubated at 30° for 18 hr. pH value throughout the experiment:  $7.5 \pm 0.1$ . ○—○, levan produced; ●—●, sucrose hydrolysed; △—△, multiplication.

Fig. 2. Levan production, sucrose hydrolysis and multiplication as a function of glutamic acid concentration. With exception of the nitrogen source, conditions are the same as those described for Fig. 1. ○—○, levan produced; ●—●, sucrose hydrolysed; △—△, multiplication.

Similar results were obtained when a defined mineral solution was used instead of tap water (Table 1); when peptone was replaced by glutamic acid, levan formation significantly decreased. This was possibly due to the presence of trace elements in the peptone and thus the tap water medium may be the more correct one for comparing peptone with other nitrogen sources. It may be seen from Figs. 1 and 2 that while sucrose hydrolysis and growth were parallel, levan formation did not follow growth and was depressed at the higher nitrogen concentrations. If the amounts of sucrose hydrolysed and levan formed are calculated/unit weight of organism, it is found that the rate



of sucrose hydrolysis remains approximately constant, while levan formation is considerably decreased.

Table 1. *Levan production, sucrose hydrolysis and multiplication of corynebacterium in the presence of different concentrations of nitrogen*

Defined mineral solution served as the mineral source. Sucrose concentration: 100 mg./ml. Incubation time: 18 hr. pH value throughout the experiment:  $7.5 \pm 0.1$ .

	Nitrogen source (mg./ml.)	Levan produced (mg./ml.)	Sucrose hydrolysed (mg./ml.)	Multiplication (organisms/ml.; $\times 5 \times 10^8$ )
	None	1.0	2.9	1.0
Peptone	0.2	21.0	21.9	2.8
	0.5	20.1	23.1	4.0
	1.0	19.8	24.0	4.9
	10.0	6.1	49.5	9.0
Glutamic acid	0.1	14.0	10.7	1.3
	0.2	12.5	12.0	1.6
	0.5	11.7	12.3	2.3
	1.0	9.8	12.5	2.7
	10.0	4.2	14.8	4.0

*Levan synthesis as a function of time.* The results of these experiments are shown in Figs. 3 and 4, where it is seen that the inhibition in levan formation in the higher nitrogen concentrations began from the early hours of the experiments.

*Effect of various concentrations of sucrose and peptone on levan synthesis.* This experiment (Table 2) was carried out to elucidate the importance of the sucrose/peptone ratio on levan formation. It may be concluded that levan synthesis is not affected by that ratio.

Table 2. *Levan production, sucrose hydrolysis and multiplication of a corynebacterium in the presence of different concentrations of peptone and sucrose*

In addition to peptone and sucrose, the medium contained phosphate buffer M/30, pH 7.6 in tap water and was inoculated to contain  $5 \times 10^8$  organisms/ml. Determinations were made after 18 hr. of incubation.

Composition of the medium		Sucrose/ peptone ratio	Levan produced (mg./ml.)	Amount of suc. converted to levan* (%, w/v)	Sucrose hydrolysed (mg./ml.)	Multiplication (organisms/ml. $\times 5 \times 10^8$ )
Sucrose (mg./ml.)	Peptone (mg./ml.)					
100.0	0.2	500	22.1	22.1	27.8	3.0
100.0	1.0	100	20.0	20.0	33.5	5.0
100.0	10.0	10	6.3	6.3	58.0	9.8
50.0	0.2	250	10.9	21.8	14.7	2.7
50.0	1.0	50	11.0	22.0	20.4	4.0
50.0	10.0	5	3.2	6.4	27.2	8.0
10.0	0.2	50	2.5	25.0	4.9	3.2
10.0	1.0	10	2.1	21.0	5.8	4.7
10.0	10.0	1	1.0	10.0	8.1	9.5
5.0	0.2	25	1.0	20.0	3.0	3.2
5.0	1.0	5	1.0	20.0	3.0	5.2
5.0	10.0	0.5	0.8	17.0	3.4	10.0

\* Maximal theoretical yield: 50 %.

*Experiments with cell-free extracts.* Organisms were grown on the media detailed in Table 3 and the cell-free extracts examined for levansucrase and sucrase activities. The results are summarized in Table 3. Sucrase activity of

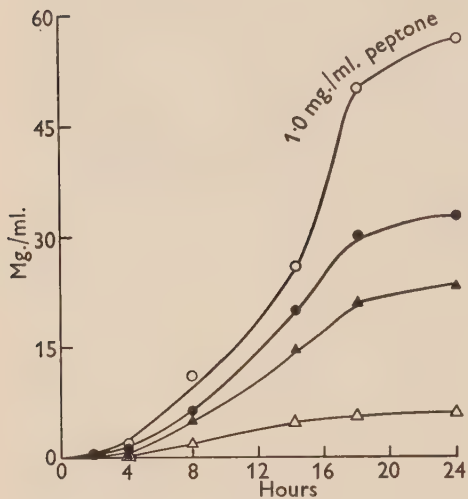


Fig. 3

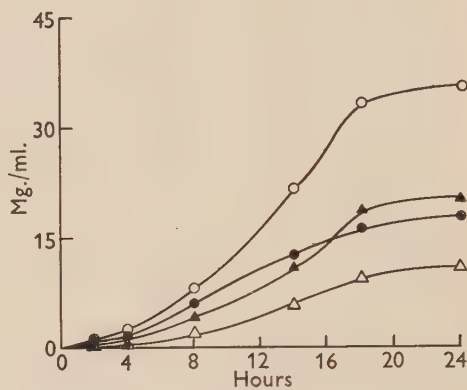


Fig. 4

Fig. 3. Levansucrase and sucrase hydrolysis as a function of time (nitrogen source—peptone). Conditions are the same as those described for Fig. 1. 0.1 ml. were taken at intervals. ▲—▲, levansucrase produced in the presence of 0.25 mg. peptone/ml.; △—△, levansucrase produced in the presence of 1.0 mg. peptone/ml.; ●—●, sucrase hydrolysed in the presence of 0.25 mg. peptone/ml.; ○—○, sucrase hydrolysed in the presence of 1.0 mg. peptone/ml.

Fig. 4. Levansucrase and sucrase hydrolysis as a function of time (nitrogen source—glutamic acid). With exception of the nitrogen source, conditions are the same as those described for Fig. 1, 0.1 ml. were taken at intervals. ▲—▲, levansucrase produced in the presence of 0.25 mg. glutamic acid/ml.; △—△, levansucrase produced in the presence of 1.0 mg. glutamic acid/ml.; ●—●, sucrase hydrolysed in the presence of 0.25 mg. glutamic acid/ml.; ○—○, sucrase hydrolysed in the presence of 1.0 mg. glutamic acid/ml.

Table 3. *Levansucrase and sucrase activity of cell-free extracts obtained from a corynebacterium grown under different conditions*

Cell-free extracts were obtained from organisms grown on the following media: (1) A medium composed of phosphate buffer M/30, pH 7.6, 0.30 mg. peptone/ml. and 100 mg. sucrose/ml. in tap water. (2) The same, with 10 mg. peptone/ml. instead of 0.30 mg./ml. (3) A medium containing 50 mg. glucose/ml. instead of 100 mg. sucrose/ml. (4) As no. 1, without peptone. The reaction mixtures contained 0.5 mg. protein/ml., 50 mg. sucrose/ml. and M/45 phosphate buffer (pH 7.0). They were incubated at  $-37^{\circ}$  for 2 hr.

Medium for growth	Activity of cell-free extracts	
	Levansucrase synthesized (mg./ml.)	Sucrase hydrolysed (mg./ml.)
No. 1	5.10	9.20
No. 2	0.82	1.94
No. 3	0.054	0.042
No. 4	0.071	0.061

the cell-free extracts was decreased compared with that of the intact organisms grown in the presence of high nitrogen concentration.

*Effect of pH value on levansucrase activity.* The results are given in Fig. 5. Optimal activity was obtained at pH 7.0; the shape of the pH activity curve is asymmetric.

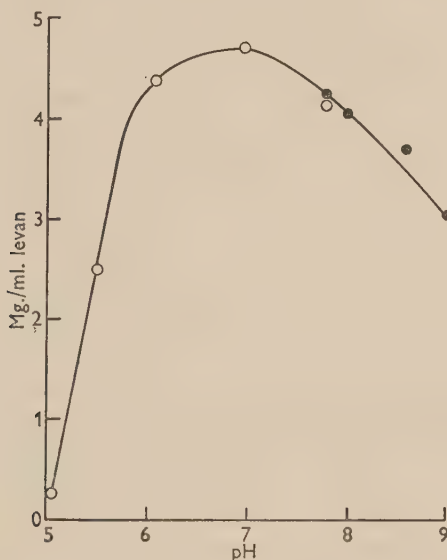


Fig. 5. Effect of pH on the activity of levansucrase obtained from a *Corynebacterium* sp. The reaction mixture contained 0.5 mg. protein/ml., 50 mg. sucrose/ml. and phosphate or borate buffer in distilled water. 3 ml. of the reaction mixture were incubated at 37° for 2 hr. ○—○, Sørensen phosphate buffer M/45; ●—●, KCl-borate-NaOH buffer M/30.

*Levan-splitting activity of cell-free extracts.* Cell-free extracts were examined for levanase (Hehre, 1955) and levanpolyase (Hestrin & Goldblum, 1953) activity. Purified levan was incubated with cell-free extracts obtained from organisms grown in low and high concentrations of nitrogen. The reaction mixtures contained 10 mg. levan/ml., 0.5 mg. protein/ml. and M/45 phosphate buffer (pH 7.0). Reaction mixtures were incubated at 37°. Mixtures of cell-free extracts and levan in buffer were used as controls. In an experiment which lasted for 24 hr., four samples were taken at 2 hr. intervals and a fifth after 24 hr. When examined by paper chromatography, no traces of oligo or monosaccharides could be detected.

## DISCUSSION

The dependence of levansucrase formation on the presence of an inducer (sucrose) and an exogenous nitrogen source (peptone or glutamic acid), leads to the conclusion that this enzyme is adaptive (see review by Karstrom, 1938). An adaptive levansucrase also exists in *Bacillus subtilis* and in *B. polymyxa*. The only bacterium known to have a constitutive levansucrase is *Aerobacter*

*levanicum* (Hestrin, Avineri-Shapiro & Aschner, 1943). Avineri-Shapiro & Hestrin (1945) described a levansucrase preparation obtained from *A. levanicum* which showed optimal activity in pH values of 5.0–5.8; the shape of the pH activity curve was shown to be symmetric. A similar levansucrase preparation was obtained from *B. subtilis* by Doudoroff & O'Neal (1945). The levansucrase preparation obtained from the corynebacterium described by us, differs from those preparations in its behaviour towards changes in pH values (Fig. 5). The decrease of levansucrase formation by the corynebacterium studied here in the presence of a high nitrogen concentration needs further consideration. Similar phenomena were observed by Beijerinck (1912) concerning dextran production by a *Leuconostoc* sp. and by Duguid & Wilkinson (1953) when investigating polysaccharide formation by *Aerobacter aerogenes*. The suggestion by Beijerinck that this phenomenon is due to decrease in the pH of the medium during growth cannot be accepted here. The suggestion by Duguid & Wilkinson (1953) that the decrease in polysaccharide formation in *A. aerogenes* is due to the disappearance of the substrate (lactose) as a result of its use by the organisms as an energy source does not meet the present case. Apparently the decrease in polysaccharide formation by the corynebacterium in high nitrogen concentrations is due to the inhibition of enzyme formation. There might also be other factors which inhibit levan formation, such as the presence of glucose in high concentration, which inhibits enzyme action (Hestrin & Avineri-Shapiro, 1944). Such a factor cannot be important here as inhibition starts when the amount of reducing sugar is still small and the amounts of reducing sugar both in the presence of low and high nitrogen concentrations are of the same order. Competition might exist between two enzyme-forming systems (levansucrase and sucrase) inside the organisms due to the presence of the common inducer (sucrose). If in higher nitrogen concentrations sucrase is formed before levansucrase and sucrase formation can be induced by lower concentrations of sucrose, then sucrose which penetrated the organism might be hydrolysed before it could induce levansucrase formation. This, however, is not the case, since sucrase activity/unit wt. of organism remained approximately constant both in low and high concentrations of nitrogen. A working hypothesis to explain this phenomenon involves the possible interconversion between levansucrase and sucrase or other enzyme system. In testing this hypothesis, certain difficulties are unavoidable. It is uncertain how much sucrose is hydrolysed by levansucrase itself since preparations of this enzyme from other bacteria are known to hydrolyse sucrose (Hehre, 1955). Another difficulty is our failure to prepare enzyme solutions, from organisms grown in high nitrogen concentration, which will show the same relation between sucrase and levansucrase activities as do the growing organisms. Short-period experiments with resting organisms might be helpful in trying to solve this problem.

I wish to express my gratitude to Professor M. Aschner under whose direction this work was performed, for his generous advice. I should also like to thank Miss Dinah Godinger for technical assistance.



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## Trace Metal Requirements and some Enzyme Systems in a Riboflavin-requiring Mutant of *Neurospora crassa*

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**SUMMARY:** A mutant of *Neurospora crassa* is shown to have an absolute requirement for riboflavin when grown at 30°, but this requirement is less stringent when the organism is grown at 25°. The Fe, Cu, Zn, Mn and Mo requirements of the mutant, grown at either temperature, are similar to those of the wild type (146) so that it seems unlikely that these metals are involved in the biosynthesis of riboflavin. A study of enzyme patterns in the mutant, grown at 30° and given optimal or deficient concentrations of riboflavin, demonstrated alternative pathways of electron transfer in the fungus. When riboflavin is deficient, the iron enzymes are markedly increased and oxygen is probably the main terminal acceptor of the electrons. At optimal concentrations of riboflavin, the flavoprotein enzymes are produced and nitrate and nitrite reductases are active so that nitrate can act as a terminal acceptor. Iron deficiency is readily produced in the mutant when riboflavin is deficient because of the increased activity of iron enzymes; a molybdenum requirement is greater at optimal riboflavin concentrations because of the enhanced production of molybdo-flavoproteins.

Mitchell & Houlahan (1946) showed that in a temperature-sensitive mutant of *Neurospora crassa* a riboflavin requirement shown during growth at 30° was eliminated by growing the fungus at 25°. In the present investigation the trace metal requirements of this mutant grown at the two temperatures were determined as well as some of the enzyme changes which occurred when the fungus was grown at 30° with optimum or deficient concentrations of riboflavin.

### METHODS

*Mutant.* The riboflavin-requiring mutant of *Neurospora crassa* which is temperature-sensitive was kindly supplied by Dr A. Mitchell, California Institute of Technology, U.S.A.

*Culture medium.* The mutant was grown in the following medium: sucrose, 20 g.;  $\text{NH}_4\text{NO}_3$ , 20 g.; Na tartrate, 1 g.;  $\text{KH}_2\text{PO}_4$ , 3 g.;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5 g.; NaCl, 0.1 g.;  $\text{CaCl}_2$ , 0.1 g.; biotin, 5  $\mu\text{g}$ .;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $9.6 \times 10^{-4}$  g.;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $8.8 \times 10^{-3}$  g.;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $2.7 \times 10^{-4}$  g.;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $7.2 \times 10^{-5}$  g.;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ ,  $1.2 \times 10^{-5}$  g.; distilled water, 1 litre.

*Purification of culture media.* The methods used to remove trace metals from the macronutrients were described elsewhere (Nicholas, 1952). The micronutrient elements, Fe, Cu, Zn, Mn and Mo were as 'spectroscopically pure' compounds supplied by Johnson & Matthey (Hatton Garden, London, E.C. 1). Biotin (25  $\mu\text{g}$ .) dispensed in phosphate buffer (pH 7) was supplied in ampoules by the Ashe Laboratories (Leatherhead, Surrey). Riboflavin dispensed in glass-distilled water in an amber bottle was stored in the dark at 0°. The culture

medium (100 ml.) was dispensed in 500 ml. Erlenmeyer flasks, and after sterilizing at 10 lb./sq.in. for 15 min. and cooling, these were inoculated with a mycelial suspension of the mutant in glass-distilled water. Before inoculating and incubating the flasks at 30°, riboflavin was added aseptically (25 µg./100 ml.) when optimal growth of the mutant was required. The organism was grown for 4 days at either temperature.

*Preparation of cell-free extracts.* The mycelial mats collected in a Büchner funnel were washed thoroughly with glass-distilled water and were frozen for 3 hr. at -17°. They were then homogenized in three times their weight of cold 0.1 M-phosphate buffer (pH 7.5) with a pestle and mortar and then in a Ten Broeck glass macerater at 0°. The homogenate was centrifuged at 2000 g for 10 min. at 4°. *Tris*-(hydroxymethyl)-aminomethane buffer 0.1 M (pH 7.5) was used to extract the 'phosphate' enzymes. The homogenates were dialysed for 12 hr. against the same buffers in which they had been prepared.

*Cofactors and other compounds.* Diphosphopyridine nucleotide (DPN) of 50% purity was prepared from baker's yeast by the method of Kornberg (private communication). Triphosphopyridine nucleotide (TPN) 95% purity and cytochrome *c* were obtained from Sigma Chemical Company, U.S.A., and riboflavin-5-phosphate (FMN) from Nutritional Biochemicals Corporation, U.S.A. Reduced DPN was prepared enzymically by the alcohol dehydrogenase method of Pullman, Colowick & Kaplan (1954) and reduced TPN by using the isocitric dehydrogenase enzyme from acetone powder of pig heart. Boiled pig heart extract, centrifuged at 18,000 g for 20 min., at 4° was used as a source of flavine adenine dinucleotide (FAD).

#### *Enzymes assayed*

*Catalase activity* was measured at 37° by the perborate method of Feinstein (1949). The reaction mixture contained 8 ml. 1.5% (w/v) NaBO<sub>3</sub>·4H<sub>2</sub>O (pH 6.8); 1.5 ml. 0.1 M-phosphate buffer (pH 6.8); 0.1 ml. enzyme extract; and was incubated at 37° for 5 min. Then 10 ml. 2 N-H<sub>2</sub>SO<sub>4</sub> was added and the solution titrated with 0.05 N-KMnO<sub>4</sub>. The values are expressed as mmole perborate degraded in 5 min./mg. protein.

*Peroxidase* was determined by the spectrophotometric method of Smith, Robinson & Stotz (1949). The reaction mixture contained 1.3 ml. of McIlvaine's buffer (pH 6.0); 1.0 ml. 10<sup>-3</sup> M-reduced 2:6-dichloroindophenol; 0.5 ml. 0.1 M-H<sub>2</sub>O<sub>2</sub>; 0.1 ml. enzyme extract. The oxidation of the dye was followed at 625 mµ. at 15 sec. intervals. The unit of activity is the change in log. *I*<sub>0</sub>/*I* of 0.001/min. calculated between 15 and 75 sec./mg. protein.

*Cytochrome oxidase* was measured in homogenates of mycelial felts by the method of Smith & Stotz (1949). The reaction solution contained 1.5 ml. 0.2 M-phosphate (pH 6.8); 1.0 ml. 10<sup>-3</sup> M-reduced 2:6-dichloroindophenol; 0.5 ml. 1 × 10<sup>-4</sup> M-cytochrome *c*; 0.2 ml. enzyme extract. The units of activity corrected for endogenous rate, are similar to those for peroxidase.

*TPN-cytochrome c reductase* was measured at 551 mµ. The reaction mixture contained: 2.5 ml. 0.1 M-phosphate buffer (pH 7.5); 0.5 ml. oxidized cytochrome *c*, 3 × 10<sup>-4</sup> M; 0.1 ml. TPNH (2 µmole/ml.) 0.1 ml. enzyme extract.



*DPNH and TPNH oxidases* ('diaphorase' systems) were measured by following dye reduction at 625 m $\mu$ . The reaction mixture used was as follows: 2.5 ml. 0.1 M-phosphate (pH 7.5); 0.3 ml.  $1 \times 10^{-3}$  M-2,6-dichloroindophenol; 0.1 ml. 0.1 M-KCN; 0.1 ml. DPNH (10 mg./ml.) or TPNH (2  $\mu$ mole/ml.); 0.1 ml. enzyme extract.

*Nitrate reductase* was measured by the method of Nicholas & Nason (1954). The test procedure consisted of adding 0.1 ml. enzyme extract to a solution containing 0.1 ml. 0.1 M-KNO<sub>3</sub>; 0.04 ml.  $10^{-3}$  M-FMN; 0.02 ml.  $10^{-3}$  M-KCN; 0.04 ml.  $2 \times 10^{-3}$  M-TPNH and 0.30 ml. 0.1 M-phosphate (pH 7.5). After 10 min. incubation at 25°, 3.5 ml. water and 0.5 ml. 1% (w/v) sulphanilamide reagent and 0.5 ml. 0.001% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride naphthylethylenediamine reagent were added. After 10 min. the test solutions were read on the Spekker absorptiometer at 540 m $\mu$ . Control tests without TPNH were used to correct for the turbidity of the enzyme. Units of activity are expressed as  $\mu$ mole NO<sub>2</sub> formed/mg. protein in 10 min.

*Nitrite reductase* was determined by a modification of the method described by Nason & Zucker (1956). The test consisted of adding 0.1 ml. enzyme to a solution containing 0.1 ml.  $10^{-3}$  M-KNO<sub>2</sub>; 0.05 ml. boiled pig heart extract; 0.05 ml. DPNH (10 mg./ml.) and 0.2 ml. 0.1 M-phosphate buffer (pH 7.5). After 10 min. incubation at 25° the colour was developed and determined as in the nitrate reductase assay.

The following enzymes were examined in 0.1 M-*tris*-(hydroxymethyl)-aminomethane (pH 7.5) extracts of the mycelial felts.

*Alkaline phosphatase* was estimated by the method of Bessey, Lowry & Brock (1946) in which the breakdown of *p*-nitrophenylphosphate to *p*-nitrophenol was measured. The enzyme activity is expressed as  $\mu$ mole P released in 30 min./mg. protein.

*Hexokinase* was measured by the method of Crane & Sols (1953). To 5 ml. solution containing ATP, MgCl<sub>2</sub> and bromothymol blue at  $2.5 \times 10^{-3}$  M;  $5 \times 10^{-3}$  M and 0.0003% (w/v) respectively is added 0.8 ml. 5% glucose; 0.2 ml. enzyme extract and the solution adjusted to pH 7.4. The control cell contained water instead of the enzyme. The change in colour was measured at 5 min. intervals for 30 min. at 620 m $\mu$ . by means of a spectrophotometer. The enzyme activity is expressed as the change in  $\log I_0/I$  of 0.001 calculated between 5 and 25 min./mg. protein.

*Phosphorylase* was measured by the method of Whelan (1955). The reaction mixture contained 0.2 ml. 5% (w/v) soluble starch; 0.5 ml. 0.5 M-citric acid-NaOH buffer (pH 6.0); 0.1 ml. enzyme extract; 1.7 ml. water equilibrated at 35° for 10 min. and then 1 ml. 0.1 M-glucose-1-phosphate added. The reaction was stopped after 10 min. by adding 5 ml. 5% (w/v) trichloroacetic acid and after centrifuging at 3000 g for 10 min. a sample was taken for P determination. Control tubes were incubated with acid added at the beginning of the reaction. The activity is expressed in  $\mu$ mole P formed/mg. protein.

*Phosphoglucomutase* was determined by the method of Najjar (1955). The reaction mixture contained 0.1 ml. 0.1 M-MgSO<sub>4</sub>; 0.1 ml. 0.1 M-glucose-1-phosphate and 0.1 ml. 0.1 M-cysteine hydrochloride (pH 7.5) freshly prepared, which



was equilibrated at 30° before adding 0.2 ml. enzyme extract. The solution was incubated for 10 min. and then 1 ml. 5 N-H<sub>2</sub>SO<sub>4</sub> added and the volume made to 5 ml. with distilled water. The reaction tubes were put in a boiling water bath to hydrolyse the remaining glucose-1-P. The control tubes were treated with H<sub>2</sub>SO<sub>4</sub> as above at zero time. P was determined in 1 ml. of the reaction mixture. The activity is expressed in  $\mu$ mole P/mg. protein or  $\mu$ mole glucose-6-P formed/mg. protein.

Protein was determined by the biuret method of Robinson & Hogden (1940).

## RESULTS

### *Riboflavin requirements*

Fig. 1 shows the effect of riboflavin on the growth of the mutant. At 30° there was no growth unless riboflavin was supplied; at 25° appreciable growth occurred without added riboflavin. Maximum yield of the organism was however obtained when the fungus was grown at 30°.

### *Trace metals in relation to growth*

A comparison between the trace metal requirements of *Neurospora crassa* wild type 146 and the riboflavin-requiring mutant grown at 25 and 30° is made in Fig. 2. Irrespective of temperature, Fe, Zn, Cu, Mo and Mn were all required for optimal growth of the mutant. The dry-weight yields of the mutant were similar to those of the wild type 146 when both were grown at 30°, but these were greater than the weights obtained when the mutant was grown at 25°. The micronutrient requirements for optimal growth of the wild type 146 and of the mutant grown at 25° or 30° were similar, being as follows ( $\mu$ g./100 ml. medium): Fe, 15; Zn, 25; Mo, 1; Cu, 2; and Mn, 2.

### *Enzymes*

Enzyme assays were made in extracts of the mutant grown at 30° at optimal and deficiency concentrations of riboflavin (50 % decrease of weight of fungus). The results for the iron enzymes are given in Table 1. Riboflavin deficiency resulted in a significant increase in peroxidase and cytochrome oxidase. Cytochrome *c* reductase, which has flavin adenine dinucleotide in the prosthetic group, was markedly less in amount when riboflavin was limiting. Catalase was only slightly depressed.

The results for the flavoprotein enzymes are listed in Table 2. The four flavo-protein enzyme systems were less active in riboflavin-deficient mycelium. The nitrate and nitrite reductase enzymes were more markedly decreased in amount than were the diaphorase systems, so that the riboflavin available appeared to be preferentially utilized in the diaphorase systems. The addition of boiled pig heart extract, a source of FAD, restored the activity of these enzymes (except nitrate reductase), almost to the level of controls, in extracts of deficient felts.

The results of assays for four phosphate enzymes are given in Table 3. Hexokinase activity was about 30 % higher in riboflavin deficient mycelium. The other three enzymes appear to be unaffected by riboflavin deficiency.

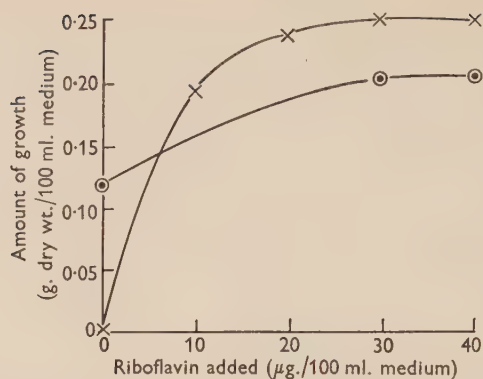


Fig. 1. Effect of additions of riboflavin on growth of 'riboflavin-requiring' mutant of *Neurospora crassa*. ○—○ growth at 25°; ×—×, growth at 30°.

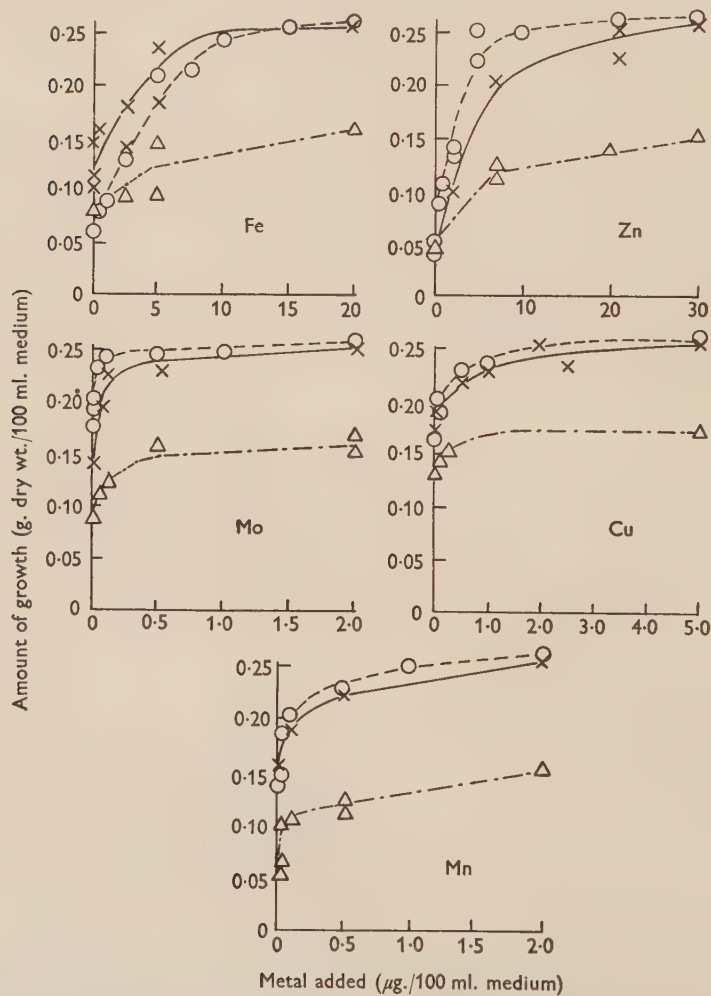


Fig. 2. Effect of trace metals on growth of wild type (146) and 'riboflavin requiring' mutant of *Neurospora crassa*. ○---○, wild type at 30°; △---△, mutant at 25°; ×—×, mutant at 30°.

Table 1. *Effect of riboflavin deficiency on the iron enzymes in the mycelium of Neurospora crassa mutant grown at 30°*

Enzyme	Expt. no.	Normal mycelia	Riboflavin-deficient mycelia
		Units of enzyme activity/mg. protein	
Catalase	1	1160	812, 70 % of normal
	2	1475	1244, 85 % of normal
	3	1494	1256, 84 % of normal
Peroxidase	1	38	91, 240 % of normal
	2	54	83, 154 % of normal
	3	150	409, 270 % of normal
Cytochrome <i>c</i> oxidase	1	300	464, 155 % of normal
	2	117	148, 127 % of normal
	3	663	804, 121 % of normal
Cytochrome <i>c</i> reductase	1	275	92, 33 % of normal
	2	145	38, 26 % of normal
	3	180	60, 33 % of normal

Table 2. *Effect of riboflavin deficiency on flavoprotein enzymes in the mycelium of Neurospora crassa mutant grown at 30°*

(Mean of 4 experiments)

Enzyme	A	B	FAD added <i>in vitro</i> to extracts of riboflavin deficient mycelia	
	Normal	Riboflavin-deficient mycelia		
	Units of enzyme activity/mg. protein		B as % of A	(% of A)
DPNH diaphorase	975	485	50	93
TPNH diaphorase	783	242	31	79
Nitrate reductase	19.4	2.0	10	10
Nitrite reductase	15	2.5	16	94

Table 3. *Effect of riboflavin deficiency on 'phosphate' enzymes in the mycelium of Neurospora crassa mutant grown at 30°*

Enzyme from	Phosphatase	Hexokinase	Phosphorylase	Phosphoglucomutase
	Units of enzyme/mg. protein			
Control mycelium	1.65	181	1.09	2.14
Riboflavin-deficient mycelium	1.50	180	1.08	1.83

## DISCUSSION

A temperature-sensitive mutant of *Neurospora crassa* is shown to have an absolute requirement for riboflavin when grown at 30°, but this is markedly less exacting when grown at 25°. The reason for the different requirement for the vitamin at the two temperatures cannot be explained in terms of a decrease in amount of flavoprotein enzymes in the fungus grown at 25°. In fact there

is no difference between the activity of the flavoprotein enzymes in the mutant grown at the two temperatures so that riboflavin must be synthesized from its precursors in the fungus at 25° (unpublished result).

There is a similar requirement for Fe, Zn, Cu, Mn and Mo in the wild type 146 and for the mutant grown at 25° or 30°. It seems unlikely therefore that these metals are involved in the biosynthesis of riboflavin in the mutant.

The results of enzyme assays of the mutant grown at 30° with optimal and deficient concentrations of riboflavin demonstrate alternative mechanisms for electron transport in the mould. When riboflavin is deficient two iron enzymes (peroxidase and cytochrome oxidase) are produced in large amounts and oxygen is probably the terminal electron acceptor because the flavoprotein enzymes, nitrate and nitrite reductases, are considerably diminished in quantity. The TPNH and DPNH diaphorase systems and cytochrome *c* reductase, which are flavin dependent, are also much decreased in amount when riboflavin is deficient. Iron deficiency is more readily produced in riboflavin-deficient mycelium, when the iron enzymes are produced in quantity, and the Mo requirement is greater when the riboflavin concentration is optimal, the flavoprotein enzymes then being very active. Thus the electron transfer mechanism of this organism involves an iron system when riboflavin is deficient and a molybdoflavoprotein system when the riboflavin concentration is optimal. The effects of Fe and Mo deficiencies on electron transfer in the fungus are similar to those reported for *Pseudomonas fluorescens* by Lenhoff, Nicholas & Kaplan (1956).

The effect of FAD (boiled pig heart) or FMN in reactivating the DPNH and TPNH diaphorase systems and nitrite reductase in extracts of riboflavin-deficient mycelium shows that their apoenzymes are unaffected by riboflavin deficiency. A shortage of riboflavin did not affect the flavoprotein enzymes to the same degree; thus nitrate and nitrite reductases are more markedly decreased in amount than are the DPNH or TPNH diaphorase systems. This may be explained in terms of a greater affinity of the apoenzyme of some flavoproteins for the flavin nucleotides available; the ones having the greatest affinity would retain their activity longest.

Thanks are due to Miss Elizabeth Atkinson who assisted with the preparation of pure cultures of the mutant and with the determination of the enzymes in the fungus described in this paper.

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## A Biochemical-taxonomic Study of a Marine Micrococcus, *Gaffkya homari*, and a Terrestrial Counterpart

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**SUMMARY:** The morphology, minimal nutritional requirements, and biochemical reactions of the lobster pathogen, *Gaffkya homari*, and of a tetracoccus found contaminating meat products were compared. The marine and terrestrial cocci were identical by the *in vitro* methods used. The validity of the genus *Gaffkya* is discussed. The 4 strains of micrococci herein studied had an unusual nitrogen requirement: all required glutamic acid as nitrogen source; substrates were not utilized in the absence of glutamate.

Snieszko & Taylor (1947) described a Gram-positive, tetrad-forming micrococcus from septicæmic lobsters and called it *Gaffkya homari*. W. S. Sturges (1950, personal communication) isolated Gram-positive, tetrad-forming micrococci as occasional contaminants of meat products in a mid-western United States meat-packing plant. In the present study two strains each of the marine tetracoccus and of the terrestrial tetracoccus, were examined biochemically and morphologically. No clear differences were detected between them. This uniformity made it necessary to examine the criteria used to define the genus *Gaffkya*.

### METHODS

**Organisms.** *Gaffkya homari* (ATCC 10400) was obtained from the American Type Culture Collection; *G. homari* RT-4 was obtained from Dr J. S. Getchell, Agricultural Experiment Station, Orono, Maine. The terrestrial tetrad-forming micrococci strain S-1 was isolated from the exposed cut surface of bologna sausage and strain S-5 was isolated from a discoloured area of meat, and were obtained through the courtesy of Dr W. S. Sturges, Cudahy Packing Co., Omaha, Nebraska. *G. tetragena* (ATCC 159) was obtained from Mr S. A. Rosenthal, Department of Bacteriology, University of Maine, Orono, Maine. *Micrococcus sodonensis* was obtained from Dr A. Bicknell, Michigan State College, East Lansing, Michigan; *Sarcina lutea* from Dr J. Webb, Biology Department, City College, New York, N.Y.; *Staphylococcus flavocyaneus* from the National Collection of Type Cultures (NCTC 7011).

The culture methods and the measurement of growth were essentially those used by Baker, Sobotka & Hutner (1953). Cultures were usually incubated at 35-37° for 5-7 days. Stock cultures were maintained on a medium described by Aaronson (1955). All chemicals were obtained from commercial sources.

Diagnostic tests for the identification of micrococci were those recommended in the Manual of Methods published by the Society of American Bacteriologists.

## RESULTS

*Morphology.* These marine and terrestrial micrococci are Gram-positive, non-motile, *c.* 0.8–1.0  $\mu$ . diam., and form tetrads; their form is unchanged after several years on artificial media. On agar slopes and plates they grow as minute greyish white raised colonies (*c.* 1.0 mm. diam.) with rough surfaces and entire edges. They usually appear as beads rather than streaks or sheets on slopes; the rapid formation of acid may prevent confluent growth. In a chemically defined medium buffered with 0.5 % glutamic acid + 0.5 % *Tris*-(hydroxymethyl)-aminomethane and initially at pH 8.7, the pH value will be lowered below 5.0 by 5 days of growth.

*Diagnostic biochemical reactions.* All 4 strains gave the same diagnostic reactions by the tests used (Table 1). For comparison the reactions of other cocci are included (see also Table 2).

*Response to antibiotics.* As noted in Table 2, the differences between *Gaffkya homari* ATCC 10400 and S-1 were minor as compared with the difference between these organisms and the other cocci.

*Minimal nutritional requirements.* All 4 strains grew well in a chemically defined medium (Table 3). Final growth, however, was stimulated as much as 20 % after the addition of a mixture of amino acids (Table 4).

Sucrose, glycerol, glucose or gluconate served as both carbon and energy source, provided that a small amount of glutamic acid (*c.* 0.1 %, w/v) was present. Fumarate, succinate, lactate, citrate, acetate, glycine, L-glutamate, DL-aspartate, DL-asparagine, and DL-alanine did not support growth even with added glutamic acid.

Sulphate satisfied the sulphur requirement. In a defined medium the optimal pH was 8.7 (the highest pH value tested). The optimum temperature for growth was *c.* 35°.

*Nitrogen requirement.* All 4 strains had an absolute requirement for glutamic acid; it was not adequate as the sole source of energy, carbon and nitrogen but supported growth on the addition of suitable carbon and energy sources. Attempts to replace the glutamate with  $\text{NH}_4\text{Cl}$ ,  $\text{KNO}_2$ ,  $\text{KNO}_3$ , glycine, DL-alanine, DL-aspartate, DL-asparagine, and L-glutamine (added aseptically), each at several concentrations within the range 0.05–1.0 % (w/v) failed.

*Osmotic and salt tolerances.* All 4 strains were similar in their responses to increasing osmotic pressure and increasing salt concentration (Table 5). Pentaerythritol did not inhibit any of the 4 strains in the concentration range used (1.0–10.0 %).

## DISCUSSION

*Comparison of marine and terrigenous strains.* The consistent similarities noted here argue that the strains should all be denoted as *Gaffkya homari* despite their diverse origins. The occurrence of related or identical species of bacteria in the sea and on the land has previously been noted (Wood, 1952; ZoBell, 1946). The marine and terrestrial environments of the cocci herein studied may be much alike in respect to osmotic pressure and high

Table 1. *Diagnostic biochemical reactions of Gaffkya homari strains and other cocci*

Strains	...	...	<i>G. homari</i>		Terrestrial strains	
			ATCC 10400	RT-4	S-1	S-5
Arabinose			Acid	Acid	Acid	Acid
Dulcitol			n.c.	n.c.	n.c.	n.c.
Glucose			Acid	Acid	Acid	Acid
Inositol			n.c.	n.c.	n.c.	n.c.
Lactose			Acid	Acid	Acid	Acid
Maltose			Acid	Acid	Acid	Acid
Mannitol			Acid	Acid	Acid	Acid
Sucrose			Acid	Acid	Acid	Acid
Xylose			n.c.	n.c.	n.c.	n.c.
Tryptone broth (indole)			n.c.	n.c.	n.c.	n.c.
Starch hydrolysis			n.c.	n.c.	n.c.	n.c.
Gelatin hydrolysis			n.c.	n.c.	n.c.	n.c.
Litmus milk			n.c.	n.c.	n.c.	n.c.
NO <sub>3</sub> reduced to NO <sub>2</sub>			n.c.	n.c.	n.c.	n.c.
Urea broth			n.c.	n.c.	n.c.	n.c.
Citrate broth			n.c.	n.c.	n.c.	n.c.

Strains	...	...	<i>G. tetragena</i> ATCC 159	<i>Micrococcus</i> <i>sodonensis</i>	<i>Sarcina</i> <i>lutea</i>	<i>Staphylococcus</i> <i>flavocyaneus</i>
Arabinose			n.t.	n.t.	n.t.	n.t.
Dulcitol			n.t.	n.t.	n.t.	n.t.
Glucose			n.c.	n.c.	n.c.	n.c.
Inositol			n.t.	n.t.	n.t.	n.t.
Lactose			n.c.	n.c.	n.c.	n.c.
Maltose			n.c.	n.c.	n.c.	n.c.
Mannitol			n.c.	n.c.	n.c.	n.c.
Sucrose			n.c.	n.c.	n.c.	n.c.
Xylose			n.t.	n.t.	n.t.	n.t.
Indole			n.c.	n.c.	n.c.	n.c.
Starch			n.c.	n.c.	n.c.	n.t.
Gelatin			+	n.c.	+	+
Litmus milk			Basic	n.c.	Basic	n.t.
NO <sub>3</sub> →NO <sub>2</sub>			+	n.c.	n.c.	n.c.
Urea broth			n.t.	n.t.	n.t.	n.t.
Citrate broth			+	+	n.c.	n.c.

n.c.=no change; n.t.=not tested; +=growth or change in medium.

Table 2. *Sensitivity of cocci to several antibiotics*

The organisms were grown on nutrient agar.

	<i>G. homari</i> ATCC 10400	<i>G. homari</i> S-1	<i>G. tetragena</i> ATCC 159	<i>Micrococcus</i> <i>sodonensis</i>	<i>Staphylococcus</i> <i>flavocyaneus</i>	<i>Sarcina</i> <i>lutea</i>
	Inhibition zone diameter (cm.) average of two experiments					
Streptomycin, c. 500 units/disk	2.9	2.7	4.1	3.3	4.3	4.5
Penicillin G, c. 500 units/disk	4.3	3.7	5.1	5.0	6.2	6.0
Aureomycin, c. 250 $\mu$ g./disk	3.5	3.6	3.2	2.2	4.4	4.1
Polymyxin, c. 500 units/disk	1.0	0.9	1.0	1.1	1.1	1.3



Table 3. *Defined medium for strains of Gaffkya homari*

Na <sub>2</sub> glycerophosphate.5H <sub>2</sub> O	0.1 g.	Ca pantothenate*	0.2 mg.
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.05 g.	Thiamine HCl*	0.2 mg.
NH <sub>4</sub> Cl	0.05 g.	Nicotinic acid*	0.2 mg.
L-Glutamic acid*	0.5 g.	Biotin*	1.0 µg.
Sucrose	1.0 g.	Mo (as Na <sub>2</sub> Mo <sub>7</sub> O <sub>4</sub> .2H <sub>2</sub> O)	0.8 mg.
Glycerol	0.5 g.	Ca (as chloride)	1.0 mg.
Tris(hydroxymethyl)aminomethane	0.5 g.	Trace metals solution§	1.0 ml.
DL-Leucine†	5.0 mg.	Distilled water to 100 ml.;	
Hypoxanthine*‡	2.0 mg.	final pH value 8.5-8.8	

\* Required.

† Stimulatory but not required.

‡ Purines may be replaced by *p*-aminobenzoic acid, folic acid or folinic acid

§ Formula in Aaronson (1955).

Table 4. *Amino acid supplement which stimulated growth when added to the defined medium of Table 3*

	g.		g.
DL-Alanine	0.04	DL-Methionine	0.006
L-Arginine (free base)	0.03	DL-Phenylalanine	0.004
DL-Aspartic acid	0.05	L-Proline	0.004
L-Glutamic acid	0.1	DL-Serine	0.01
Glycine	0.05	DL-Threonine	0.01
L-Histidine (free base)	0.02	DL-Tryptophan	0.005
DL-Isoleucine	0.005	L-Tyrosine	0.004
DL-Leucine	0.005	DL-Valine	0.005
DL-Lysine HCl	0.045	Distilled water to 100 ml.;	
		final pH value 6.5.	

Table 5. *Effect of osmotic pressure and salt concentration on strains of Gaffkya homari grown on a chemically defined medium. See Table 3*

		Strain			
		ATCC 10400	RT-4	S-1	S-5
Salt conc.		Growth expressed as optical density.			
% (w/v)					
0		1.08	1.04	0.83	0.75
NaCl	1.0	0.46	0.70	0.78	0.98
	3.0	0.72	0.77	0.67	0.86
	5.0	0.82	0.68	0.66	0.72
	7.0	0.64	0.73	0.74	0.67
	10.0	0.02	0.06	0	0.03
NaNO <sub>2</sub>	0.1	0.52	0.71	0.52	0.44
	0.3	0.32	0.64	0.40	0.38
	1.0	0.12	0.30	0.28	0.21
	3.0	0.02	0.18	0.12	0.02
	5.0	0	0	0	0
	7.0	0	0	0	0
NaNO <sub>3</sub>	1.0	1.30	0.94	1.00	0.90
	3.0	1.08	0.98	1.00	0.97
	5.0	0.83	0.84	0.66	0.89
	7.0	0.98	0.80	0.77	0.90
	10.0	0.76	0.61	0.64	1.00

concentrations of electrolytes and of organic compounds. These environmental similarities may account for the success in isolating similar strains. It would be of interest to know whether the strains isolated from meat products can cause septicæmia in lobsters.

*Glutamic acid as an obligate nitrogen source.* The strains studied have a requirement for glutamic acid as a nitrogen source which is neither spared or replaced by any of the nitrogen-containing compounds tested. Gilvarg & Davis (1954) reported an obligate glutamate requirement for an *Escherichia coli* mutant. Wiame & Storck (1953) reported that glutamate would serve as the sole nitrogen source for certain *Bacillus subtilis* mutants; several amino acids replaced the glutamate. As experiments with  $\alpha$ -ketoglutarate were not tried with *Gaffkya homari* strains, it is not clear whether or not the specificity of this requirement resides in the carbon skeleton.

*Critique of the genus Gaffkya.* As defined by *Bergey's Manual* (1948) *Gaffkya* strains '...occur in animal body and in special media as tetrads, while in ordinary culture media they occur in pairs and irregular masses. Aerobic to anaerobic. Gram positive. Parasitic organisms.' The lobster pathogen fits this genus since it is a tetrad-forming Gram-positive coccus and a parasite. Parasitism seems to be an important—even essential—criterion for assigning a coccus to *Gaffkya*. If one followed *Bergey's Manual*, the saprophytic strains S-1 and S-5 would be excluded from *Gaffkya*. The strains S-1 and S-5 are nutritionally, biochemically and morphologically identical with the strains of *Gaffkya homari* by all the *in vitro* methods here used; these strains must be assigned to *G. homari* if the criterion of parasitism be abandoned.

The nomenclatural vicissitudes of the genus *Gaffkya* attest to the inadequacy of its definition. It was created by Trevisan (1885) for the tetrad coccus isolated by Gaffky (1883) and called by him *Micrococcus tetragenus*. The Winslows (1908) placed this species in the genus *Albococcus* with the white staphylococci. Buchanan (1917) placed it in *Staphylococcus*, Hucker (1924*a*, 1928) and Fleming (1929) in *Micrococcus*; and *Bergey's Manual* (1930) returned it to *Gaffkya*. Lehmann & Neumann (1931) placed it in *Sarcina*, but succeeding editions of *Bergey's Manual* retained *Gaffkya* as a genus. Wood (1950), Shaw, Stitt & Cowan (1951) and Bisset (1952) advocated the elimination of *Gaffkya* because of the close similarity of these bacteria to other micrococci with which they intergrade. It would appear from the present study that the criteria used to distinguish the genus in *Bergey's Manual*, especially parasitism, are dubious; the genus may have to be dismantled and its component species distributed elsewhere, e.g. *G. tetragena* may have to be placed in *Staphylococcus* (*Micrococcus*) occupying a position alongside *S. albus*, with which it shares colonial morphology, habitat, and antigens (Hucker, 1924*b*, Hucker & Robertson, 1926). *G. tetragena* and *G. homari* share tetrad-formation and parasitism, but they may not otherwise be closely related. They differ morphologically (tetragena cells are larger and colonial and slope growth is more abundant) and biochemically (tetragena does not ferment carbohydrates or milk and does reduce nitrate to ammonia; *Bergey's Manual*; Snieszko & Taylor, 1947) and they have no common agglutinogens (Rosenthal, 1950). Shaw *et al.* (1951)

described cocci ('group iii') which they placed among the staphylococci to which *G. homari* shows some resemblance; unfortunately their description does not permit detailed comparison.

The good agreement between the biochemical reactions and the morphological criteria currently used in bacterial taxonomy and the additional information on growth factors, carbon, nitrogen, and sulphur sources, and effects of chemical agents, including antibiotics, seen in these studies suggest that biochemical criteria may well complement the older taxonomic criteria.

I wish to thank Professor R. F. Nigrelli of New York University for his support and encouragement and Professor E. R. Hitchner of the University of Maine for his aid in obtaining some of the bacterial strains used and for stimulating discussions on the biology of the lobster infection.

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## The Production of Capsules, Hyaluronic Acid and Hyaluronidase by 25 Strains of Group C Streptococci

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**SUMMARY:** Of the 25 group C streptococci examined all but 3 produced a hyaluronidase detectable in a sensitive turbidimetric test. These 3 strains and 6 of the hyaluronidase producers were heavily capsulated on serum glucose agar and in serum + glucose enriched liquid media. The capsules of all strains were readily destroyed by testicular hyaluronidase. In continually neutralized cultures of the capsulated strains hyaluronic acid was detected only transiently during logarithmic growth with the enzyme producers but rose to a high steady concentration with the other 3 strains. Hyaluronidase activity was present throughout the phase of logarithmic growth and beyond, but fell markedly in the 16 hr. following the cessation of logarithmic growth of capsulated strains in neutralized cultures and even more markedly in cultures grown without neutralization. Non-capsulated variants of the 9 capsulated organisms resembled the parent strains in enzyme production. A single passage through mice did not qualitatively influence capsulation, hyaluronic acid or hyaluronidase production by those strains examined.

McClean (1941) reported hyaluronidase production by 5 non-capsulated group C strains; 5 other strains, 4 of them capsulated, did not produce the enzyme. Crowley (1944) found that 35 of 55 non-capsulated group C strains of human origin produced hyaluronidase. Both these workers used the mucin clot prevention (MCP) test. More recently Di Caprio, Rantz & Randall (1952) found that 5 group C strains tested produced high titres of hyaluronidase by the MCP method. Murray & Pearce (1949), who measured enzyme activity by the degree of decapsulation of mucoid streptococci on solid media, examined 8 group C strains, only one of which produced hyaluronidase. Russell & Sherwood (1949), using a viscosimetric method, reported hyaluronidase formation by 2 group C strains among 10 examined.

The production of hyaluronidase by a capsulated group C streptococcus in quantities too small for detection by the MCP test was described by MacLennan (1956*a*). It was thought worthwhile therefore to examine hyaluronidase production by other capsulated group C strains to see if they behaved similarly. Some non-capsulated strains were also examined by the sensitive turbidimetric test (Pike, 1948*a*; MacLennan, 1956*a*) since it seemed unlikely that the methods of Murray & Pearce (1949) and of Russell & Sherwood (1949) were more sensitive than this test.

### METHODS

*Source of strains.* National Collection of Type Cultures (NCTC).

*Cultivation of organisms.* This has been fully described elsewhere (MacLennan, 1956*a*). Cultures were grown from heavy inocula (saline washed) in

serum + glucose enriched broth at 37°, with or without continual neutralization by sodium hydroxide.

*Measurement of hyaluronic acid and hyaluronidase.* Hyaluronic acid present in culture supernatant fluids was estimated by acid serum precipitation (Pike, 1946; MacLennan, 1956*a*). Hyaluronidase was estimated by a similar precipitation of residual hyaluronic acid after incubation of culture supernatant fluids with added polysaccharide for 16 hr. at 37° at pH 7.2–7.4 in the presence of 1/10,000 thiomersalate (MacLennan, 1956*a*). Quinn, Seastone & Weber (1953) reported that the measurement of hyaluronic acid by the turbidimetric method of Pike (1946) was subject to error when continually neutralized cultures were used. This, they said, was due to an increase in the buffer capacity of the cultures as neutralization proceeded, leading to a decrease in the turbidity produced by hyaluronic acid with acid serum which might be misinterpreted as being caused by hyaluronidase. It was occasionally observed here with capsulated group C strains in the late log phase of growth that precipitation of the hyaluronic acid in supernatants with acid serum produced finely turbid precipitates which rapidly became granular, then flocculent, and finally sedimented. These precipitates were denser to the naked eye than the normal fine stable precipitates and yet gave lower colorimeter readings. Normal turbidities could, however, be obtained by exhaustively dialysing the late log phase culture supernatant fluids against uninoculated culture medium before precipitation. Although this behaviour before dialysis leads to considerable inaccuracy in hyaluronic acid estimations it certainly does not sufficiently explain the decrease in turbidity in ageing cultures; this also occurred when these cultures were incubated with a bacteriostatic agent without further neutralization and it did not occur at low temperature (MacLennan, 1956*a*) or, as will be shown, in heated cultures. The decrease in turbidity under these conditions cannot be due to an increase in buffer capacity.

*Capsulation.* This was best demonstrated on organisms cultivated for 24 hr. on plates of moist 20 % horse serum + 1 % glucose agar. UNO ink was used as a background stain (MacLennan, 1956*a*) as suggested by Dr Nuala Crowley (personal communication). Pl. 1 shows the even backgrounds obtained with this ink. The clear spaces in the background are due to capsular material, they are absent from smears of hyaluronidase-treated cultures.

## RESULTS

All strains were plated on moist serum glucose agar following revival in blood broth from NCTC dried cultures. Sixteen strains gave non-mucoid colonies alone, the remaining 9 strains gave a mixture of mucoid and non-mucoid colonies which were separated by plating single colonies and found to breed true by serial subculture on agar. These differences in colony form were less readily obvious on fresh blood agar. On this medium all strains except 4669 were haemolytic; two strains, 4670 and 4671, gave  $\alpha$ -haemolysis.

Capsulated organisms were demonstrated only in mucoid colonies, and

suspensions of these organisms in saline were completely decapsulated by 5 min. incubation with 50 MCP units testicular hyaluronidase/ml. saline suspension. Heated hyaluronidase was inactive. As others have suggested this indicates that hyaluronic acid is an essential part of these capsules. Pl. 1 shows the degrees of capsulation of the 9 capsulated strains on serum glucose agar. Rather surprisingly, perhaps, in view of the influence of anaerobiosis on the capsulation of strain 6176 in broth culture (MacLennan, 1956*a*) the cultivation of all 9 capsulated strains on serum glucose agar under completely anaerobic conditions did not decrease capsulation.

Hyaluronic acid estimations were made on continually neutralized, shake cultures of the 9 capsulated strains, after the elimination of uncapsulated variants, and of two non-capsulated strains. Cultures were sampled at intervals, centrifuged, and the supernatant fluids precipitated with acid serum. Three capsulated strains produced hyaluronic acid which reached a maximum concentration in the culture supernatant fluids by the end of growth (Table 1) and was unaltered by further incubation at 37° for 3 days. The cultures of the capsulated strains 6176 and 4675, on the other hand, and of the 2 non-capsulated strains did not contain hyaluronic acid at any of the times of sampling. Although the hyaluronic acid was demonstrated in cultures of strain 6176 by more frequent sampling the rise and fall in concentration was always more rapid than that observed in earlier work (MacLennan, 1956*a*) with this strain. It seems likely that small differences in the composition of the medium might upset the balance of hyaluronic acid synthesis and destruction sufficiently to account for this difference in behaviour. The remaining 5 capsulated strains showed an increase in concentration of hyaluronic acid during growth, followed by a decrease similar to that previously described for strain 6176 and shown to be due to hyaluronidase action (MacLennan, 1956*a, b*).

Table 1. *Hyaluronic acid production by group C streptococci*

Strain	Capsules	Culture ages (hr.)					
		2	4	5	6	9	24
		Mg. hyaluronic acid/100 ml. culture					
NCTC 6178	0	0	0	0	0	0	0
NCTC 6176 non-mucoid	0	0	0	0	0	0	0
NCTC 6176 mucoid	+	0	0	0	0	0	0
NCTC 6177	+	0	0	7	20	0	0
NCTC 6180	+	0	0	5	10	0	0
NCTC 4675	+	0	0	0	0	0	0
NCTC 4676	+	0	0	0	10	0	0
NCTC 7912	+	0	0	0	5	0	0
NCTC 6963	+	0	5	10	20	40	40
NCTC 7022	+	0	5	7	20	24	56
NCTC 7023	+	0	5	7	10	20	56

Hyaluronidase estimations on the culture supernatant fluids of the 6 capsulated enzyme-producing strains and 2 non-capsulated strains revealed enzymic activity in young cultures and throughout active growth.



Hyaluronidase was present after 2 hr. growth although capsulation of 6 of the strains was still noted after 4 hr. The time of appearance of hyaluronidase in culture supernatant fluids was earlier and the activity apparently greater than with group A organisms (Pike, 1948*b*; Quinn *et al.* 1953, Faber & Rosendal, 1954).

There was a distinct decrease in hyaluronidase activity in the 16 hr. following the cessation of active growth. This decrease was due, in part at least, to the thermolability of the hyaluronidase, as with strain 6176 (MacLennan, 1956*b*). Supernatant fluids from late logarithmic-phase cultures of the 6 capsulated hyaluronidase-producing strains were adjusted carefully to pH 7.2, heated for 1 and 2 days at 37° in the presence of 1/10,000 thiomersalate, then tested for hyaluronidase; there was a considerable decrease in activity. Supernatant fluids heated at 54° for 2 hr. were completely inactive, further confirming the enzymic nature of the hyaluronic acid destruction.

The influence of the rapid decrease of pH value upon the hyaluronidase activity of unneutralized cultures containing glucose was examined. Rogers (1945) noted a suppression of hyaluronidase production in cultures of a group C streptococcus due to acid formation, and Hale (1944) showed that streptococcal hyaluronidase was rapidly inactivated at pH 4.6. Therefore the supernatant fluids from cultures of the 26 strains grown with neutralization until the end of logarithmic growth and also from cultures grown for 24 hr. without neutralization were tested for hyaluronidase. The 24 hr. supernatant fluids were adjusted to pH 7.2–7.4 before testing for hyaluronidase. The non-capsulated variants of the mucoid strains and a selection of strains passaged once in mice were also tested. Table 2 summarizes the results. All non-capsulated strains, whether grown with neutralization or not, completely destroyed the added hyaluronic acid; heating the supernatant fluids at 54° for 2 hr. prevented this destruction. On the other hand, although hyaluronidase-producing capsulated strains partly or completely destroyed added hyaluronic acid when neutralized cultures were used, the supernatant fluids from 24 hr. unneutralized cultures showed little or no activity. Since this was true also of non-capsulated variants of these 9 strains, there was no suggestion that the absence of capsulation was due to increased hyaluronidase synthesis, in agreement with previous work with strain 6176 (MacLennan, 1956*a*).

A single mouse passage of a number of strains by the intraperitoneal route causing death within 24 hr. did not qualitatively influence the production of capsules, hyaluronic acid or hyaluronidase (Table 2).

#### DISCUSSION

By a sensitive test more group C streptococci have now been shown to produce hyaluronidase than has previously been reported. In addition to using a sensitive test, early sampling and continual neutralization are important for the demonstration of hyaluronidase production by capsulated organisms and their non-capsulated variants but not for that produced by non-capsulated group C strains. The time of appearance of the enzyme in cultures of capsulated



Table 2. The production of capsule, hyaluronic acid and hyaluronidase by group C streptococci

0, ±, +, ++, denote the extent to which the substance listed at the head of the columns is produced. A and B refer to estimations made on logarithmic-phase neutralized cultures before and after mouse passage respectively. Column C records the enzyme activity of 24 hr. cultures grown without neutralization.

Strain	Non-mucoid						Mucoid					
	Hyaluronic acid			Hyaluronidase			Hyaluronic acid			Hyaluronidase		
	A	B		A	B	C	A	B		A	B	C
NCTC 6963	±	.		0	.	.	+	+	+	0	0	.
NCTC 7022	±	.		0	.	.	+	+	+	0	0	.
NCTC 7023	±	.		0	.	.	+	+	+	0	0	.
NCTC 6176	.	.		++	+	0	+	+	0	+	+	0
NCTC 6177	.	.		+	.	0	+	+	+	+	+	0
NCTC 6180	.	.		+	.	0	+	+	+	+	+	0
NCTC 4675	.	.		+	.	±	0	+	+	+	+	0
NCTC 4676	.	.		+	.	0	+	+	+	+	+	0
NCTC 7912	.	.		+	.	0	+	+	+	+	+	0
NCTC 6178	.	.		+	+	+	+	+	+	.	.	.
NCTC 4540	.	.		+	+	+	+	+	+	.	.	.
NCTC 5370	.	.		+	+	+	+	+	+	.	.	.
NCTC 6179	.	.		+	+	+	+	+	+	.	.	.
NCTC 6181	.	.		+	+	+	+	+	+	.	.	.
NCTC 7136	.	.		+	+	+	+	+	+	.	.	.
NCTC 8544	.	.		+	+	+	+	+	+	.	.	.
NCTC 8546	.	.		+	+	+	+	+	+	.	.	.
NCTC 7855	.	.		+	+	+	+	+	+	.	.	.
NCTC 9413	.	.		+	+	+	+	+	+	.	.	.
NCTC 9414	.	.		+	+	+	+	+	+	.	.	.
NCTC 4670	.	.		+	+	+	+	+	+	.	.	.
NCTC 5371	.	.		+	+	+	+	+	+	.	.	.
NCTC 4669	.	.		+	+	+	+	+	+	.	.	.
NCTC 4671	.	.		+	+	+	+	+	+	.	.	.
NCTC 7201	.	.		+	+	+	+	+	+	.	.	.

strains is earlier and the activity apparently greater than with group A organisms (Pike, 1948*b*; Faber & Rosendal, 1954), although differences in culture media and size of inoculum may partly account for this.

With capsulated strains which produce hyaluronidase, hyaluronic acid was either detected only over a short period of culture growth or not at all. This is probably due more to a rapid destruction of hyaluronic acid by hyaluronidase than to gross variation in the amount of hyaluronic acid synthesized, since some of these strains are as well capsulated on solid media as three strains which do not produce hyaluronidase and liberate large amounts of hyaluronic acid in broth culture.

The appearance of capsules on solid or liquid media can be completely suppressed by cultivation in the presence of added hyaluronidase (McClean, 1941; Murray & Pearce, 1949); it is therefore possible that some non-capsulated, hyaluronidase-producing streptococci also produce hyaluronic acid but that, because of the production of more hyaluronidase than in capsulated strains, a capsule is never formed, even on solid media. The report that a penicillin-resistant variant of a non-mucoid hyaluronidase-producing group A streptococcus (Faber & Rosendal, 1955) produced hyaluronic acid supports this idea, particularly as there was an inhibition of release of hyaluronidase from this variant organism during the exponential growth phase. On the other hand, since the non-capsulated variants of 3 capsulated strains resemble their parents in not producing hyaluronidase and since there is no detectable increase in enzyme production by non-capsulated variants of capsulated, hyaluronidase-producing strains (Table 2), the absence of hyaluronic acid and capsules from some strains is clearly due to inability to synthesize the polysaccharide.

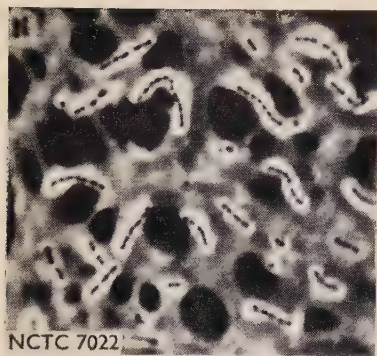
I wish to thank Dr D. McClean for his interest in this work, Dr I. A. Macpherson for taking photographs and Mr D. C. Hawkins for technical assistance.

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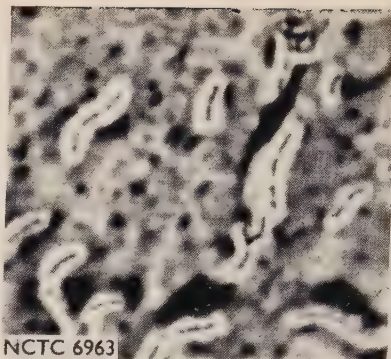




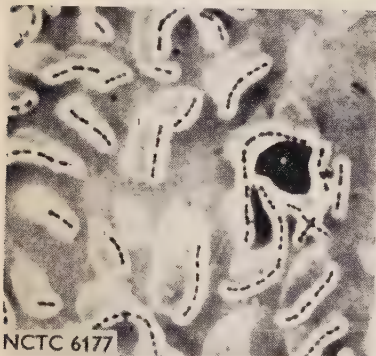
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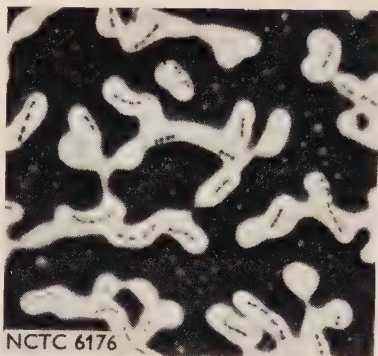
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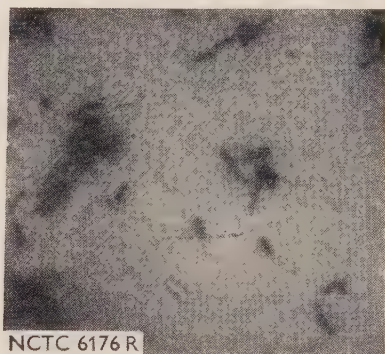
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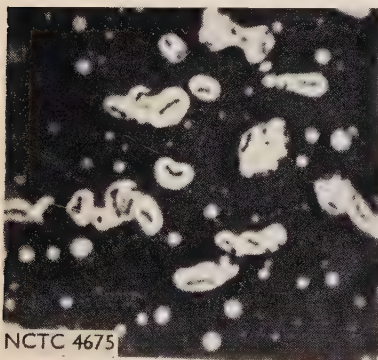
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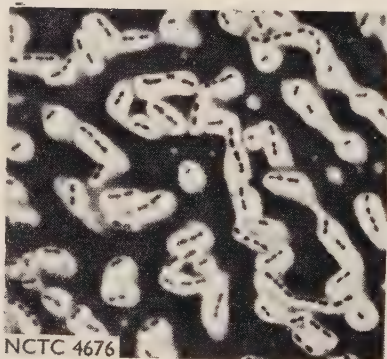
NCTC 6176



NCTC 6176 R



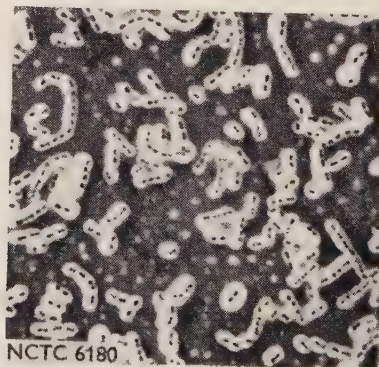
NCTC 4675



NCTC 4676



NCTC 7912



NCTC 6180

A. P. MacLENNAN—PRODUCTION OF CAPSULES, HYALURONIC ACID AND  
HYALURONIDASE BY 25 STRAINS OF GROUP C STREPTOCOCCI

(Facing p. 491)



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#### EXPLANATION OF PLATE

Capsule production by group C streptococci. All strains except 7022, 7023 and 6963 produce hyaluronidase. 6176R is a non-capsulated variant of 6176. All strains were grown on 20 % (v/v) horse serum + 1 % (w/v) glucose nutrient agar for 24 hr.

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## Growth Rate and Generation Time of Bacteria, with Special Reference to Continuous Culture

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**SUMMARY:** The relations between growth rate, generation time distribution and age distribution in growing bacterial cultures are derived. The effect of inheritance on generation time is probably negligible. Some applications to experimental data exemplify the mathematical results. The validity of the principal assumptions is discussed.

It appears to be tacitly assumed by many bacteriologists, in particular by some who are concerned with the study of continuous cultures, that there is a simple relationship between growth rate and generation time. It is sometimes stated that during the phase of steady exponential growth of a batch culture the following equation holds:

$$\frac{d \log N}{dt} = \nu = \frac{\log 2}{\bar{\tau}}, \quad (1)^\dagger$$

where  $N$  (supposed very large) is the number of organisms in the culture,  $\nu$  is the number growth rate constant, and  $\bar{\tau}$  is the mean generation time (in a sense not clearly defined). Equation (1) is false except in special cases that are apparently never realized in practice. Similarly, in the steady state of a continuous culture running at dilution rate  $D$  it is usually false that

$$D = \frac{\log 2}{\bar{\tau}}. \quad (2)$$

The true relationships, though more subtle, differ quantitatively from (1) and (2) by very little, but so far as (2) is concerned, that little is an expression of the selective power which is so important a feature of continuous culture.

In this paper I describe some simple quantitative features of bacterial populations which result from the dispersion of the individual generation times. I assume (i) that the populations under discussion are homogeneous, i.e. that they consist of organisms of only one type, and that the cultures have continued in steady growth long enough for the age distribution to have become constant; (ii) that the numbers of organisms present are so large that variations can be regarded as continuous, and statistical fluctuation as negligible. Some of the mathematical results are not new, but they are somewhat inaccessible to bacteriologists, and I have given simple derivations, glossing over many difficulties which the pure mathematician must attend to, but which are resolved by observation for the bacteriologist.

To avoid the use of inappropriate or ambiguous phrases, I adopt the following terminology: the 'inception' and 'termination' of an organism are

† Natural logarithms are used throughout.

respectively the events by which it becomes a recognizably separate entity (by fission of its parent) and by which it ceases to be so (by itself dividing). The same words may be used to mean the epochs of these events. The 'age' of an organism (to be distinguished from the age of a culture) is then the time which has elapsed since its inception; a 'young' organism is one whose age is small compared with the mean generation time.

#### Mass and number growth rate

If in a batch culture  $x$  and  $n$  are the mass and number of organisms, respectively, per unit volume, we can write for the phase of 'logarithmic' growth

$$\frac{dx}{dt} = \mu x, \quad (3a)$$

$$\frac{dn}{dt} = \nu n, \quad (3b)$$

where  $\mu$  and  $\nu$  are the mass and number growth rates. If the population density is not too large, so that the organisms are well nourished,  $\mu$  and  $\nu$  take constant values  $\mu_m$  and  $\nu_m$  which are characteristic of the medium. In a well-balanced medium, thoroughly aerated, it appears possible to grow some organisms at a constant rate for about thirty generations, i.e. until the inoculum has multiplied  $10^9$ -fold. Thus Powell (1956*a*) from observations on *Aerobacter cloacae*† growing on a virtually unlimited medium under the microscope, found the growth rate to be  $0.0143 \text{ min}^{-1}$ ; Dr D. Herbert (private communication), working with the same organism and medium on a larger scale, found the growth rate to be sensibly the same up to a population density of  $c. 10^{10}/\text{ml}$ .

Clearly,  $\mu$  and  $\nu$  must have the same value during steady growth, since otherwise the size of the organisms would increase or decrease indefinitely.

The mass growth rate may be written as a function of nutrilit concentration. In the simplest case, when growth is limited by a deficiency of a single substance, all others being present in excess, we have approximately

$$\mu = \mu_m \frac{s}{K + s}, \quad (4)$$

where  $s$  is the concentration of limiting nutrilit, and  $K$  is a constant, the 'saturation constant'. Usually  $K$  is exceedingly small, so that  $\mu \simeq \mu_m$  except when  $s$  is of the same order of magnitude as  $K$ , or less (cf. Monod, 1942; Dagley & Hinshelwood, 1938). In the experiments already quoted, Herbert found that growth and oxygen uptake ceased quite abruptly when the medium was exhausted of glucose. Although (4) has no real theoretical basis, it agrees with observation so far as present experimental techniques can determine.

† This organism (NCTC 8197) was formerly given the specific name *aerogenes* by the National Collection of Type Cultures. The same strain was called *Bacterium aerogenes* in Powell (1955) and *Aerobacter aerogenes* in Powell (1956*a*).

Provided, then, that the culture is adequately nourished, we can write for (3b)

$$\frac{dn}{dt} = \nu_m n,$$

or

$$n = n_0 e^{\nu_m(t-t_0)},$$

where  $n_0$  is the (number) concentration at time  $t_0$ . The growth may be characterized by a quantity  $\bar{\tau}_e$ , the 'doubling time' or 'mean effective generation time', instead of by  $\nu_m$ ; this is the time required for the number to double:

$$2n_0 = n_0 e^{\nu_m \bar{\tau}_e},$$

$$\bar{\tau}_e = \frac{\log 2}{\nu_m}.$$

(It is sometimes convenient to distinguish a mass 'doubling time',  $\bar{\tau}_d$  (cf. Herbert, Elsworth & Telling, 1956) related to  $x$  as  $\bar{\tau}_e$  is to  $n$ . The reciprocals of  $\bar{\tau}_e$  and  $\bar{\tau}_d$  are called 'taux de naissance' or 'taux de croissance' by French authors.)

#### *The distribution of generation times*

The generation times of individual organisms are far from uniform; the scatter appears to depend on the growth medium, and is on the whole greater in multicellular than in unicellular species (Powell, 1955, 1956*b*). Examples are:

(i) (Powell, 1955.) *Bacillus mycoides* growing on tryptic meat broth at 35°:

Estimated mean generation time	28.7 min.
Estimated standard deviation	14.2 min.
Coefficient of variation	0.496

(ii) (Powell, 1956*b*.) *Streptococcus faecalis* growing on tryptic meat broth at 35°:

Estimated mean generation time	26.1 min.
Estimated standard deviation	3.5 min.
Coefficient of variation	0.133

Some care is needed in defining the distribution of generation time, especially when actual measurements are envisaged (Powell, 1955). The frequency function of generation time,  $f(\tau) d\tau$ , is the probability that a newly formed organism will have a generation time in the range  $\tau$ ,  $\tau + d\tau$ . This suffices to define the distribution provided that the particular value of  $\tau$  is independent of the ancestry of the organism; I shall make the assumption in this and the next two sections. In all known examples,  $f(\tau)$  is a positively skew curve, usually leptokurtic, and with no evidence of multimodality. Various functional forms have been suggested for it (Rahn, 1932; Kendall, 1948; Kendall & Waugh, cited in Kendall, 1952; Powell, 1956*b*). Of these the Pearson type III distribution is the most convenient, and usually fits adequately:

$$f(\tau) = \frac{\tau^{g-1} e^{-\tau/m}}{m^g \Gamma(g)}. \quad (5)$$



The parameters  $g$ ,  $m$  are simply related to well-known statistics:

$$\begin{aligned} \text{Arithmetic mean of } \tau &= \mathbf{a} = gm, \\ \text{Coefficient of variation of } \tau &= c = g^{-\frac{1}{2}}. \end{aligned}$$

# *Age distribution and growth rate in batch culture*

The age distribution in a growing culture has a curious and interesting property which is not generally known; roughly speaking, the youngest organisms are present in greatest number.

To discover the distribution, write  $\phi(a)$  for the frequency function of age and consider a culture containing a large number  $N$  of organisms at time  $t=0$ , say. There are  $N\phi(a) da$  organisms whose ages are in the range  $a$ ,  $a+da$ . Now write

$$F_-(\tau) = \int_{\tau}^{\infty} f(\xi) d\xi$$

for the proportion of organisms whose generation times are greater than  $\tau$ . It can then be seen that if an organism has already attained the age  $a$ , its chance of attaining the greater age  $a+t$  without dividing is

$$\frac{F_-(a+t)}{F_-(a)}.$$

Hence of the original  $N\phi(a) da$  organisms,

$$\frac{N\phi(a) F_-(a+t) da}{F_-(a)}$$

are still extant at time  $t$ . But meanwhile the culture has grown, and now contains  $Ne^{\nu_m t}$  organisms, instead of  $N$ . The survivors then form a fraction

$$\frac{\phi(a) F_-(a+t) e^{-\nu_m t} da}{F_-(a)}$$

of the whole number, and their ages now lie in the range  $a+t$ ,  $a+t+da$ . Since the culture is by hypothesis in a steady state this fraction must be  $\phi(a+t) da$ . Thus the difference equation

$$\frac{\phi(a) F_-(a+t) e^{-\nu_m t}}{F_-(a)} = \phi(a+t) \tag{6}$$

is to hold for all  $t$ . In particular, for very small  $t$

$$F_-(a+t) = t dF_-/da + F_-(a),$$

$$\phi(a+t) = t d\phi(a)/da + \phi(a),$$

$$e^{-\nu_m t} = 1 - \nu_m t,$$

neglecting powers of  $t$  above the first. Hence, substituting in (6)

$$\begin{aligned} \frac{1}{F_-(a)} \frac{d}{da} \{F_-(a)\} - \nu_m &= \frac{1}{\phi(a)} \frac{d}{da} \{\phi(a)\}, \\ \phi(a) &= \phi(0) e^{-\nu_m a} F_-(a) = \phi(0) e^{-\nu_m a} \int_a^{\infty} f(\tau) d\tau, \end{aligned} \tag{7}$$

where  $\phi(0)$  is a constant, yet to be determined, which makes

$$\int_0^\infty \phi(a) da = 1.$$

The exponential function and the integral on the right of (7) are both positive, and decrease steadily as  $a$  increases, hence  $\phi(a)$  does so: the age distribution has a maximum at  $a=0$ . To complete this investigation we have to determine  $\phi(0)$  and the growth-rate constant  $\nu_m$  in terms of  $f(\tau)$ .

Since each fission increases the number of organisms by one, the (absolute) fission rate divided by the number of organisms present is the growth rate. The chance that an organism will attain an age  $a$  at least is  $F_-(a)$ ; the chance that it will attain age  $a+da$  at least is  $F_-(a+da)$ ,

$$=F_-(a)-f(a) da;$$

among organisms which have attained age  $a$  the fraction  $f(a) da/F_-(a)$  terminate during the succeeding short interval  $da$ ; the fission rate among such organisms is  $f(a)/F_-(a)$ . There are at any given time say  $N$  organisms in the culture, of which  $N\phi(a)$  are of age  $a$ . These contribute

$$N\phi(a) f(a) da/F_-(a)$$

to the fission rate. Taking into account organisms of all ages,

$$\begin{aligned} \nu_m &= \frac{1}{N} \int_0^\infty \frac{N\phi(a) f(a) da}{F_-(a)} \\ &= \int_0^\infty \phi(0) e^{-\nu_m a} f(a) da. \end{aligned} \quad (8)$$

Now integrate (7) by parts between limits 0 and  $\infty$ :

$$\int_0^\infty \phi(a) da = 1 = \phi(0) \left[ \frac{e^{-\nu_m a}}{-\nu_m} F_-(a) \right]_0^\infty - \phi(0) \int_0^\infty \frac{e^{-\nu_m a}}{\nu_m} f(a) da.$$

Here the last term is unity, by (8), and  $F_-(0)=1$ . Hence

$$\phi(0) = 2\nu_m.$$

Finally, we may write for the age distribution, instead of (7)

$$\phi(a) = 2\nu_m e^{-\nu_m a} \int_a^\infty f(\tau) d\tau, \quad (9)$$

while the growth rate cannot be written explicitly, but must be determined (using (8) again) by

$$2 \int_0^\infty e^{-\nu_m \tau} f(\tau) d\tau = 1 \quad (10)$$

(cf. Harris, 1951). It is evident that  $\nu_m$  cannot equal  $(\log 2)/a$  in general, though in fact the difference is quite small for unicellular organisms, the dispersion of whose generation times is moderate.

When the generation time is not dispersed, we have  $\tau=a$  for every organism and  $f(\tau)=0$  except at  $\tau=a$ . Also, by (10),  $\nu_m=(\log 2/a)$ . Then,

$$\begin{aligned} F_-(a) &= 1 \quad (a < a) \\ &= 0 \quad (a > a), \end{aligned}$$

and

$$\phi(a) = 2\nu_m e^{-\nu_m a} \quad (a < a)$$

$$= 0 \quad (a > a)$$

$$\phi(a-0) = \nu_m = \frac{1}{2}\phi(0).$$

The last equation also follows from the consideration that the number of organisms which are just about to divide must be half the number just newly formed. This special case is illustrated in Fig. 1 (curve (i)). The distributions actually found are smoothed-out versions of it; the sharpness of the fall in  $\phi(a)$  near  $a=a$  is less, the greater the dispersion of  $\tau$  (curves (ii), (iii); see also Fig. 4*a*, *b*).

The expression  $2e^{-\nu_m \tau} f(\tau)$  is everywhere positive, and so by (10) it is a frequency function. It has an important significance, more especially in continuous cultures, and it is therefore convenient to give it a distinctive name and symbol; I call it the 'carrier distribution',  $\mathcal{C}(\tau)$ . We have already seen that of the  $N\nu_m$  fissions per unit time occurring in the culture,  $N\phi(a)f(a)da/F_-(a)$  are the terminations of organisms which reach the age  $a$  but fail to reach  $a+da$ , i.e. of those whose generation times  $\tau$  lie in this interval. The distribution of the generation times of those organisms actually extant at any given time is  $f(\tau)$ , but the distribution of generation times of their immediate ancestors (mothers) is

$$\frac{N\phi(\tau)f(\tau)d\tau}{N\nu_m F_-(\tau)} = 2e^{-\nu_m \tau} f(\tau) d\tau = \mathcal{C}(\tau) d\tau. \quad (11)$$

A difficulty arises if the culture throws off some non-viable organisms. Suppose that the constant fraction  $\alpha$  of newly formed organisms are viable in the sense that they terminate within a finite time. Then the generation time distribution is such that

$$\int_0^{\rightarrow \infty} f(\tau) d\tau = \alpha,$$

and in addition there is a singular component contributing  $1-\alpha$  to the total frequency at  $\tau=\infty$ . It is not clear that viable organisms can be certainly distinguished from non-viable, but adequate conventional standards can be adopted in given circumstances. We can then if we wish define a frequency function  $f^*(\tau)$  for the generation times of viable organisms only. If  $\alpha < 1$ , the mean of  $f(\tau)$  will be infinite; that of  $f^*(\tau)$  may or may not be. Obviously for finite  $\tau$ ,  $\alpha f^*(\tau) = f(\tau)$  and we can write instead of (10)

$$1 = 2\alpha \int_0^{\infty} e^{-\nu_m \tau} f^*(\tau) d\tau.$$

Even if the mean of  $f^*(\tau)$  or  $f(\tau)$  is infinite, the integral (10) converges and gives a finite non-zero growth rate if  $\alpha > \frac{1}{2}$ . We have no right to expect that the mean is finite even in  $f^*(\tau)$ , therefore. The relatively few measurements that have been made do not in fact suggest any unusual instability in sample means such as would be symptomatic of an infinite population mean. For  $\alpha = \frac{1}{2}$  we have

$$1 = \int_0^{\infty} e^{-\nu_m \tau} f^*(\tau) d\tau = \int_0^{\infty} f^*(\tau) d\tau,$$

and  $\nu_m$  must be zero. For smaller  $\alpha$ ,  $\nu_m$  will be negative, i.e. the culture will die out. This conclusion is more readily reached by other methods (e.g. Feller, 1950). In my experience,  $\alpha > 0.99$  for well-nourished organisms (Powell, 1956*b*), and I think it may usually be assumed that  $\alpha = 1$  in batch cultures; it is easy to make a correction where it is necessary and practicable to distinguish living organisms from dead.

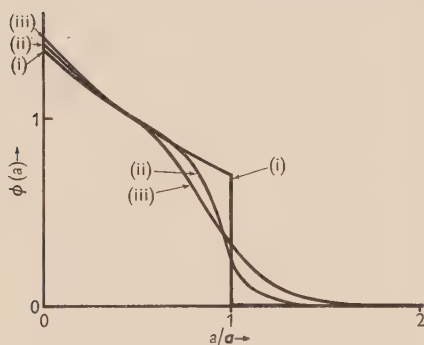


Fig. 1

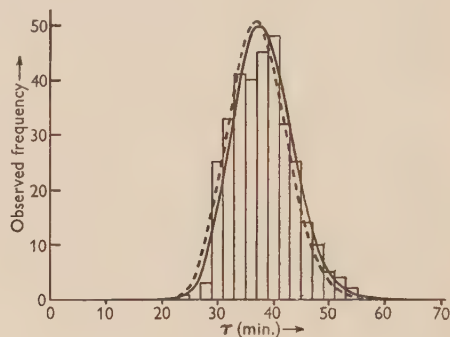


Fig. 2

Fig. 1. Effect of dispersion of  $\tau$  on age distribution, schematic. (i) No dispersion; (ii)  $f(\tau)$  a Pearson Type III with  $g=49$  ( $c=0.14$ ); (iii)  $f(\tau)$  a Pearson Type III with  $g=16$  ( $c=0.25$ ).

Fig. 2. A generation time distribution of *Pseudomonas aeruginosa*. Histogram: observed frequencies. Continuous curve: Pearson Type III distribution of best fit. Dotted curve: carrier distribution.

### Examples

(i) (Data from Powell, 1956*b*.) *Pseudomonas aeruginosa* growing in a defined medium containing glucose, glutamic acid, succinic acid, thiosulphate and the usual salts, at  $35^\circ$ :

$$a = 38.1 \text{ min.},$$

$$\text{var } \tau = 28.7,$$

$$c = 0.141.$$

The distribution was well represented by a Pearson Type III (eqn. 5), with parameters  $g=50.6$ ,  $m=0.753$  (Fig. 2). The growth rate was estimated in two ways:

(a) From eqn. (10) by insertion of (5):

$$\begin{aligned} 1 &= 2 \int_0^\infty \frac{e^{-\nu_m \tau} \tau^{g-1} e^{-\tau/m} d\tau}{m^g \Gamma(g)} \\ &= 2(1 + m\nu_m)^{-g}; \\ \nu_m &= (2^{1/g} - 1)/m. \end{aligned}$$

(b) By replacing the integral in (10) by a finite sum over the observed frequencies, using trial values of  $\nu_m$ , and interpolating:

$$(a) \text{ gave } \nu_m = 0.018314,$$

$$(b) \text{ gave } \nu_m = 0.018314,$$



while  $(\log 2)/a = 0.018189$ —a difference of 0.7% only. The carrier distribution, calculated from

$$\begin{aligned}\mathcal{C}(\tau) &= \frac{2e^{-\nu m \tau} \tau^{g-1} e^{-\tau/m}}{m^g \Gamma(g)} \\ &= \frac{2\tau^{g-1}}{m^g \Gamma(g)} \exp\left(-\frac{\tau}{m} 2^{1/g}\right)\end{aligned}$$

(again a Pearson Type III) is also shown in Fig. 2.

(ii) (New data.) *Bacillus megaterium* growing on peptone water at 35°. Observations were begun at 2.5 hr. after inoculation of spores, and were continued for 80 min. in each experiment. The experiments and reduction of the data were conducted as described in Powell (1955) for *B. subtilis* and *B. mycoides*. The sample of generation times obtained from experiments restricted to a fixed period is systematically biased, and on the assumption that the true  $f(\tau)$  is of Pearson Type III, the frequency function to be fitted to the observations is (apart from a normalizing factor)

$$\{e^{\nu m(80-\tau)} - 1\} \tau^{g-1} e^{-\tau/m} \quad (\tau \leq 80). \quad (12)$$

This curve was fitted by the method of moments (Fig. 3*a*), and the test for goodness of fit gave  $\chi^2_{12} = 18.25$ ;  $P(\chi^2) = 0.11$ . The estimates of the parameters were

$$g = 8.26,$$

$$m = 2.70;$$

whence

$$g^{-\frac{1}{2}} = c = 0.348,$$

$$mg = a = 22.3 \text{ min.},$$

$$(\log 2)/a = 0.03113 \text{ min.}^{-1},$$

$$\nu_m = 0.03247 \text{ min.}^{-1}.$$

The greater difference of the last two figures (*c.* 4.2%), as compared with the previous example, reflects the greater dispersion of  $\tau$ . From the experimental data a growth curve was constructed by adding together the number of organisms under observation at corresponding times in each experiment. It was satisfactorily linear (Fig. 3*b*), and gave another estimate of the growth rate constant

$$\nu_m = 0.03259,$$

in fair agreement with that calculated from the frequency function.

At the end of each experiment, the ages of the organisms then present were recorded, so that the age-distribution could be built up. The observed distribution was compared with that calculated from  $f(\tau)$ :

$$\phi(a) = 2\nu_m e^{-\nu_m a} I_-(a/m, g),$$

where  $I_-(a/m, g)$  is the incomplete gamma function

$$\frac{1}{\Gamma(g)} \int_{a/m}^{\infty} \xi^{g-1} e^{-\xi} d\xi.$$

(A convenient table of this function is given in Pearson & Hartley (1954). They actually tabulate  $P(\chi^2, \nu)$ :  $I_-(a/m, g) = P(2a/m, 2g)$ .) The histogram

(Fig. 4a) reproduces the general features of  $\phi(a)$ , but the fit is very bad, mainly because of the excess of observations in the cell  $a=16$ . This excess, in spite of its improbability, can only be ascribed to accident. There are two factors which increase the expectation of  $\chi^2$  in experiments of this kind: (i) there is high correlation between the generation times of sister organisms, so most observations of age occur in pairs, and the variance in each cell is nearly doubled; (ii) errors of judgement in estimating times of fission result in observations being assigned to cells adjacent to the true ones, not at random.

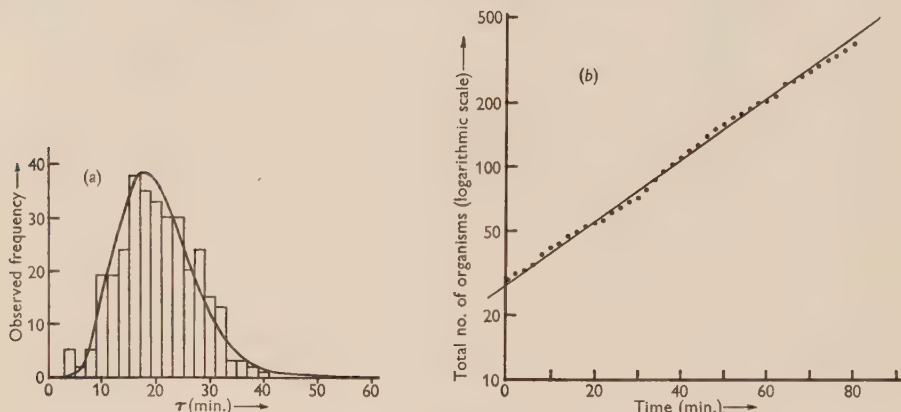


Fig. 3. (a) A generation time distribution of *Bacillus megaterium*. Histogram: observed frequencies. Continuous curve: modified Pearson Type III distribution. (b) Growth curve of *B. megaterium* under the same conditions as (a).

The median age (from either the observed or calculated distribution) is about 7 min., i.e. in a sample from such a culture half the organisms would be less than 7 min. old.

(iii) (Data from Powell, 1955.) *Bacillus mycoides* growing on tryptic meat broth at 35°. The estimated parameters of the Pearson Type III distribution were:

$$g=4.07,$$

$$m=7.04,$$

whence

$$c=0.496,$$

$$a=28.7 \text{ min.},$$

$$(\log 2)/a=0.02419 \text{ min.}^{-1},$$

$$\nu_m=0.02637 \text{ min.}^{-1}.$$

The observed and calculated age distribution are compared in Fig. 4b; the fit is fairly good ( $\chi^2_{18}=21.58$ ;  $P(\chi^2)=0.25$ ). The median is about 11 min.

#### *Age distribution and growth rate in continuous culture*

In a continuous culture maintained in a steady state, the growth rate is equal to the dilution rate,  $D$ . ( $D$  is the fraction of the volume of culture displaced by the inflowing medium, per unit time.) The generation time distribution is therefore dependent on  $D$ , and we may now write it as  $f_D(\tau)$ .

The immediate determinant of the growth rate is the concentration of the medium (or one or more constituents of it) according to some such law as (4):

$$\nu = \mu = D = \mu_m \frac{s}{K + s}. \quad (13)$$

This is true of all types of continuous culture. For simplicity, consider the case where the growth rate is restricted, if it is restricted at all, by deficiency of a single nutrilit (s), and suppose that to produce  $Y$  g. of organisms, 1 g. of this nutrilit must be consumed ( $Y$  is called the yield constant.). Then,

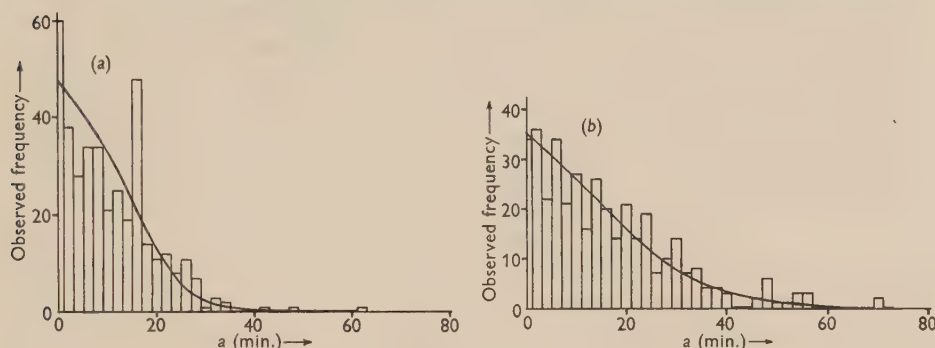


Fig. 4. (a) Age distribution of *B. megaterium* in steady growth. Histogram: observed frequencies. Continuous curve: calculated from the fitted distribution of Fig. 3a. (b) Age distribution of *B. mycoides* in steady growth. Histogram: observed frequencies. Continuous curve: calculated from the generation time distribution.

if  $x$  is the mass concentration of organisms and  $s$  the concentration of limiting nutrilit, the following equation holds at all points in the system (including medium reservoirs and receivers):

$$s_R = s + x/Y, \quad (14)$$

where  $s_R$  is the nutrilit concentration in the inflowing medium. In the 'chemostat' (Novick & Szilard, 1950) or Monod (1950) types of apparatus,  $D$  is fixed by the experimenter, and the concentration of organisms adjusts itself to satisfy (13) and (14):

$$x = Y(s_R - s) = Y \left( s_R - \frac{KD}{\mu_m - D} \right);$$

it can do so only if  $D < \mu_m s_R / (K + s_R)$ . In the 'turbidostat' (Bryson & Szybalski, 1952) type, the experimenter fixes  $x$ , and  $D$  adjusts itself to satisfy the same conditions. Here the limitation is that  $x < s_R Y$ . Novick's (1955) insistence on an essential difference between the two systems is a mistake; they have different useful ranges of applicability, but the relation of organisms to environment is the same for both, and they can be analysed in the same way up to the point at which experimental control is in question. They will not, however, behave in the same way during changes of the working conditions.

For the time being I shall assume that the mixing in the culture vessel is 'perfect', i.e. that the ingoing medium is instantaneously and homogeneously

dispersed throughout the working volume, and that the effluent is drawn equally from all points of the vessel. Then, if an organism is present at time  $t$ , the chance of its remaining in the vessel until the later time  $t_1$ , at least, is  $\exp \{-D(t_1 - t)\}$ .

All the frequency functions which have been proposed to represent the distribution of generation times can be written in the form

$$f(\tau/m; g_1, g_2, \dots) d(\tau/m),$$

where the parameters  $g_r$  are independent of  $m$ . Changes in  $m$  alter the time-scale only, and all the moments of the curves can be written as  $m^s M_s(g_1, g_2, \dots)$ . If generation times are determined by a mechanism of the Kendall (1948, 1952) or more general Kendall & Waugh type (Kendall, 1952), we might expect only the scale of the distribution to be altered under conditions of moderately restricted growth. No detailed observations have yet been made under such conditions, but it will be seen that the same principles as apply to batch culture apply here. The only change is that the growth rate  $\nu$  is now not  $\nu_m$  (in general) and is equal to  $D$ . As a reminder of the dependence on  $D$ , it is convenient to write  $\phi_D(a)$ ,  $F_{D-}(\tau)$ , as well as  $f_D(\tau)$ , where before we had  $\phi(a)$ ,  $F_-(\tau)$ .

Suppose a culture vessel contains  $N$  organisms (by hypothesis constant). At time  $t=0$ , say, there are  $N\phi_D(a) da$  organisms whose ages lie in the range  $a, a+da$ . Of this number, at time  $t$ ,

$$\frac{N\phi_D(a) F_{D-}(a+t) da}{F_{D-}(a)}$$

will still not have divided, but of the survivors only the fraction  $e^{-Dt}$  will still be in the vessel; the rest will have been washed out. Hence

$$\frac{\phi_D(a) F_{D-}(a+t) e^{-Dt}}{F_{D-}(a)} = \phi_D(a+t)$$

and, by the same reasoning as was previously used,

$$\phi_D(a) = \phi_D(0) e^{-Da} \int_a^\infty f_D(\tau) d\tau, \quad (15)$$

where  $f_D(\tau)$  is the generation time distribution under the conditions obtaining in the culture vessel.

The relation between growth rate and generation time is more simply obtained than in batch culture. The chance that an organism chosen at random will terminate in the vessel must be  $\frac{1}{2}$ . For if it terminates in the vessel, it produces two daughters; if it does not, it produces none; and the expected number of its immediate progeny must be 1 to keep  $N$  constant. The chance that its generation time is in the range  $\tau, \tau+d\tau$  is  $f_D(\tau) d\tau$ ; the chance that it remains in the vessel until it terminates is  $e^{-D\tau}$ . Thus we have

$$\frac{1}{2} = \int_0^\infty e^{-D\tau} f_D(\tau) d\tau, \quad (16)$$

formally the same as (10). It follows that  $\phi_D(0)$  in (15) is  $2D$ .

We now have a carrier distribution

$$\mathcal{C}_D(\tau) = 2e^{-D\tau} f_D(\tau); \quad (17)$$



it is not difficult to see that  $\mathcal{C}_D(\tau)$  is the generation time distribution of those organisms which remain in the vessel until their termination, i.e. of those which are responsible for the maintenance of the culture.

Equation (17) shows that a continuous culture discriminates heavily against organisms of unusually long generation time. As can be seen from Fig. 2, there is little difference between  $\mathcal{C}_D(\tau)$  and  $f_D(\tau)$  for moderate values of  $\tau$  if the dispersion is not great, but

$$\mathcal{C}_D(\tau)/f_D(\tau) = 2e^{-D\tau} \rightarrow 0 \quad \text{as} \quad \tau \rightarrow \infty.$$

Of course, by (11) the same discrimination operates in batch cultures, but the outcome is greatly complicated by the accelerations of growth which occur at the beginning and end of the 'logarithmic' phase. In continuous cultures, we should expect the population to become very quickly stable and homogeneous; organisms of large  $\tau$ , which contribute little to the growth rate, are eliminated altogether from the system. Conversely, the growth rate is affected by any persistent correlation between the generation times of related organisms (e.g. between mothers and daughters). Or, if the growth rate is fixed, the composition of the population must adjust itself. The effect of correlation is considered in the next section.

I hope to deal elsewhere with the selective property of a continuous culture as it affects the growth of contaminants and mutants.

Conditions of restricted growth such as occur when  $D$  is much less than  $\nu_m$  appear to cause the death of an appreciable proportion of the organisms (Novick, 1955; Powell, 1956*a*); it cannot then be assumed that the index of viability,  $\alpha$ , is either constant or near unity. In the steady growth of a batch culture, it can be shown that the proportion of viable organisms ( $V$ ) is  $2\alpha - 1$  (Topley & Wilson's *Principles*, 1955; the 'generation index',  $p$ , which they use is  $2\alpha$ ). The same ratio obtains in continuous cultures: Suppose the culture contains a constant number,  $N$ , of organisms of which  $NV$  are viable and  $N(1 - V)$  non-viable. During a short interval  $dt$ ,  $DN dt$  fissions occur, and with them are associated a loss of  $DN dt$  viable organisms, a gain of  $2\alpha DN dt$  viable organisms and a gain of  $2(1 - \alpha) DN dt$  non-viable. At the same time  $DN dt$  organisms are removed by the flow through the vessel; of these  $DNV dt$  are viable and  $DN(1 - V) dt$  non-viable. Hence in the steady state

$$-DN dt + 2DN\alpha dt - DNV dt = 0,$$

$$2(1 - \alpha) DN dt - DN(1 - V) dt = 0,$$

and

$$V = 2\alpha - 1,$$

$$1 - V = 2(1 - \alpha),$$

as asserted. If  $f_D^*(\tau)$  is the generation time distribution for viable organisms only, we have

$$\int_0^{\rightarrow\infty} f_D(\tau) d\tau = \alpha;$$

$$\alpha f_D^*(\tau) = f_D(\tau); \quad (\tau \text{ finite}).$$

$$F_{D-}(\tau) = \int_{\tau}^{\infty} f_D(\xi) d\xi = \int_{\tau}^{\rightarrow\infty} f_D(\xi) d\xi + (1 - \alpha),$$

where the last term represents the contribution to  $F_{D-}(\tau)$  from non-viable organisms; and

$$\mathcal{C}_D(\tau) = 2\alpha e^{-D\tau} f_D^*(\tau).$$

It is to be expected that  $\alpha$  will diminish with the medium concentration, and so with  $D$ ; this appears to be true in the few known examples which, however, have not yet been investigated systematically. There are also indications that  $\alpha$  is much reduced in over-acid media.

*Correlation between generation times: its effect on growth rate*

So far we have assumed that the generation time of an organism is independent of its ancestry; this is false, since there is high correlation between the  $\tau$  of sisters, and appreciable correlation between the  $\tau$  of cousins (and perhaps of second cousins; Powell, 1956*b*). In experiments lasting for only a short time, the mother-daughter correlation is usually found to be small or zero, but the work of Hughes (1955) suggests that an inherited tendency may persist over many generations. I am not satisfied (Powell, 1956*b*) that Hughes's conclusions follow from his results, but they can by no means be rejected at present. We have, then, to estimate the extent to which our previous calculations may be in error because of association between generation times of related organisms. The analysis becomes more difficult, and it will probably be enough to consider the two factors likely to be of major importance: correlation between mothers and daughters, and between sisters.

The following treatment applies to batch cultures; formally, the results for continuous cultures are exactly the same, the only difference being the dependence of the various frequency functions on the substrate concentration, and so on the dilution rate.

Let the product-moment correlation coefficients between generation times be  $\rho(H)$  for mothers and daughters,  $\rho(S)$  for sisters and let the joint distribution of the generation times of mothers and daughters be  $h(\tau_1; \tau_2) d\tau_1 d\tau_2$ ; this is the probability that the  $\tau$  of a mother is in the range  $\tau_1, \tau_1 + d\tau_1$  and the  $\tau$  of a given one of the daughters in  $\tau_2, \tau_2 + d\tau_2$ —we can assume that the two daughters are similarly related to the mother. Then

$$\int_0^\infty h(\tau_1; \tau) d\tau_1 = \int_0^\infty h(\tau; \tau_2) d\tau_2 = f(\tau),$$

i.e. the border distributions of  $h(\tau_1; \tau_2)$  are alike of the form  $f(\tau)$ ; but  $h(\tau_1; \tau_2)$  is not necessarily symmetrical in its arguments, though it appears to be so in practice. Also

$$\int_0^\infty \int_0^\infty \tau_1 h(\tau_1; \tau_2) d\tau_1 d\tau_2 = \int_0^\infty \int_0^\infty \tau_2 h(\tau_1; \tau_2) d\tau_1 d\tau_2 = \mathbf{a},$$

the mean generation time.

We can no longer assume that the distribution of  $\tau$  among the organisms present at any given time in the culture is  $f(\tau)$ ; for this 'population distribution' we write  $\mathcal{P}(\tau)$ , and the carrier distribution is

$$\mathcal{C}(\tau) = 2e^{-\nu_m \tau} \mathcal{P}(\tau);$$

we may also expect  $\nu_m$  to be different from the value given by (10).

We deal first with the effect of correlation between sisters. Consider the fissions occurring during a short interval  $dt$ ; these may be represented as in Fig. 5. Each fission is the inception of a pair of sisters, whose generation times, such as  $\tau_{21}$ ,  $\tau_{22}$ , may be correlated. Part of the subsequent development of each pair is shown to the right of the interval  $dt$ . Suppose now the branches of the tree to the right of  $t+dt$  are cut off at the points X, shuffled in any way, and reattached. We can arrange if we wish that there is no correlation between the members of the pairs formed. But their contribution to the

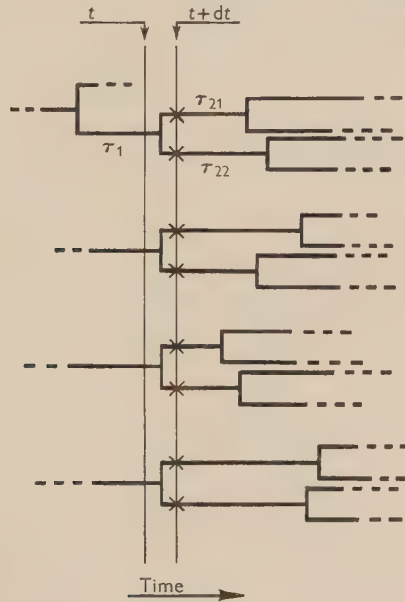


Fig. 5. Illustrating the independence of growth rate and sister-sister correlation.  $\tau_1$ ;  $\tau_{21}$ ;  $\tau_{22}$ : typical triad of mother and two daughter organisms.

growth rate of the culture remains the same; so for all intervals  $dt$ . Hence the growth rate is independent of correlation between sisters. This result is true only for steady growth.

Now  $\mathcal{P}(\tau)$  must be invariant, i.e. it must be unaltered as members of the population terminate and are replaced by others. Organisms whose generation times are in the range  $\tau_1$ ,  $\tau_1 + d\tau_1$  give rise to daughters of which the fraction

$$\frac{h(\tau_1; \tau_2) d\tau_1 d\tau_2}{f(\tau_1) d\tau_1}$$

have generation times in the range  $\tau_2$ ,  $\tau_2 + d\tau_2$ . The frequency of such mothers (Fig. 5) is  $\mathcal{C}(\tau_1) d\tau_1$ . The total contribution of daughter organisms to  $\mathcal{P}(\tau_2) d\tau_2$  from mothers of any  $\tau$  is

$$\int_0^\infty \mathcal{C}(\tau_1) \frac{h(\tau_1; \tau_2)}{f(\tau_1)} d\tau_1 = 2 \int_0^\infty e^{-\nu_m \tau_1} \frac{h(\tau_1; \tau_2)}{f(\tau_1)} \mathcal{P}(\tau_1) d\tau_1,$$

and so if  $\mathcal{P}(\tau)$  is invariant, it must satisfy

$$\mathcal{P}(\tau) = 2 \int_0^\infty e^{-\nu_m \xi} \frac{h(\xi; \tau)}{f(\xi)} \mathcal{P}(\xi) d\xi. \quad (18)$$

It is known from the theory of integral equations (see, for example, Whittaker & Watson, 1940) that

$$\mathcal{P}(\tau) = \lambda \int \Lambda(\xi; \tau) \mathcal{P}(\xi) d\xi$$

has no solution but  $\mathcal{P}(\tau) \equiv 0$ , unless  $\lambda$  has certain special values, the characteristic numbers of the nucleus  $\Lambda(\xi; \tau)$ . In our case,  $\lambda$  must be 2, but the nucleus

$$\Lambda(\xi; \tau) = e^{-\nu_m \xi} \frac{h(\xi; \tau)}{f(\xi)}$$

contains a disposable parameter  $\nu_m$  which provides room for the adjustment of the  $\lambda$ . Hence from (18) and

$$\int_0^\infty \mathcal{P}(\tau) d\tau = \int_0^\infty \mathcal{C}(\tau) d\tau = 2 \int_0^\infty e^{-\nu_m \tau} \mathcal{P}(\tau) d\tau = 1$$

both  $\mathcal{P}(\tau)$  and  $\nu_m$  can in principle be determined. We note that if the  $\tau$  of mothers and daughters are statistically independent,  $h(\tau_1; \tau_2) = f(\tau_1)f(\tau_2)$  and (18) gives

$$\mathcal{P}(\tau) \equiv f(\tau),$$

as it should.

Unfortunately, we have no guidance to the functional form of  $h(\tau_1; \tau_2)$ . The bivariate normal surface is obviously inappropriate, since both our variables are limited to positive values, and their distribution is markedly skew. The bivariate form of the 'lognormal' distribution (Johnson, 1949) would be acceptable but for its intractability in the present context, and the distributions derived by Narumi (1923*a, b*) are unsuitable for other reasons as well. In the following examples I have used a purely numerical method to obtain estimates of  $\mathcal{P}(\tau)$  in histogram form, from observed frequencies of  $f(\tau)$  and  $h(\tau_1; \tau_2)$ ; but I have not been able to prove that the iterative process converges, though it appears to do so from both sides of the apparent limit.

### Examples

(i) (Data from Powell, 1956*b*.) *Chromobacterium prodigiosum* growing on tryptic meat broth at 35°.

$$a = 22.87 \text{ min.},$$

$$\text{var } \tau = 23.34,$$

$$c = 0.211.$$

Growth rate, assuming  $\rho(H) = 0$ ,

estimated from Type III fitted function:  $\nu_m = 0.03075$ ,

estimated directly from the observations:  $\nu_m = 0.03075$ ,

$$(\log 2)/a = 0.03030.$$

Estimated  $\rho(H) = +0.302$ .

Estimated  $\rho(S) = +0.698$ .



The correlation table of the observations representing  $h(\tau_1; \tau_2)$  was slightly unsymmetrical, and the border distributions had slightly (but not significantly) different means; Bowker's (1948) test showed that the lack of symmetry was not significant. To ensure that the border distributions were exactly alike, a new table was constructed, in which the entries were

$$h_s(\tau_1; \tau_2) = \frac{1}{2}\{h_0(\tau_1; \tau_2) + h_0(\tau_2; \tau_1)\},$$

where  $h_0(\tau_1; \tau_2)$  is the observed frequency in the cell  $\tau_1; \tau_2$ . This manipulation of the data involved little disturbance, and the estimated  $\rho(H)$  for the new table was 0.299, differing from the original by much less than the sampling error. The new border frequencies were called  $f_s(\tau)$  and their sum was called  $W$ . An approximation to the solution of (18) was obtained by carrying out the following scheme of iteration:

$$\begin{cases} P'_{r+1}(\tau) = \sum_{\xi} e^{-\nu_0 \xi} \frac{h_s(\xi; \tau)}{f_s(\xi)} P_r(\xi), \\ \lambda_{r+1} = W / \sum_{\tau} P'_{r+1}(\tau), \\ P_{r+1}(\tau) = \lambda_{r+1} P'_{r+1}(\tau). \end{cases}$$

For the first step, the set  $P_0(\xi)$  was taken as  $f_s(\xi)$ , and  $\nu_0$  was taken as 0.08075, on the assumption that  $\mathcal{P}(\tau)$  did not differ much from  $f(\tau)$ . After 8 cycles, the set  $P(\tau)$  no longer changed, and  $\lambda$  was constant at 1.9913. The iteration was then repeated with better approximations to  $\nu_m$  than  $\nu_0$ . If in the integral equation

$$P(\tau) = \lambda \int_0^{\infty} e^{-\nu \xi} \frac{h(\xi; \tau)}{f(\xi)} P(\xi) d\xi,$$

$P$  is a frequency function, and we take  $\nu=0$ , we have  $\lambda=1$ , for

$$1 = \int_0^{\infty} P(\tau) d\tau = \int_0^{\infty} \lambda \int_0^{\infty} \frac{h(\xi; \tau)}{f(\xi)} P(\xi) d\xi d\tau = \lambda \int_0^{\infty} P(\xi) d\xi = \lambda.$$

But if  $\nu=\nu_m$ ,  $\lambda=2$ . Hence if we finally obtain by iteration a constant  $\lambda=\lambda_{\infty}$  with  $\nu=\nu_0$ , we might expect

$$\nu_1 = \frac{\nu_0}{\lambda_{\infty} - 1}$$

to be a better approximation to  $\nu_m$ . At the third trial, it was found that

$$\nu_2 = 0.03130$$

gave  $\lambda=1.9994$ , and this was considered sufficiently accurate. Approximately, therefore, the growth rate is

$$\nu_m = 0.0313 \text{ min.}^{-1},$$

about 2% greater than the value obtained by disregarding the mother-daughter correlation. The final set of  $P(\tau)$ , approximating to  $\mathcal{P}(\tau)$ , was used to construct the histograms in Fig. 6a, b.

(ii) (Data from Powell, 1956b.) *Bacterium (Aerobacter) cloacae* growing on tryptic meat broth at 35°.

$$\begin{aligned} a &= 24.43, \\ \text{var } \tau &= 26.92, \\ c &= 0.212. \end{aligned}$$

Growth rate, assuming  $\rho(H)=0$ ,

estimated from Type III fitted function:  $\nu_m=0.02886$ ,

estimated directly from the observations:  $\nu_m=0.02884$ ,

$(\log 2)/a=0.02838$ .

Estimated  $\rho(H)=-0.316$ .

Estimated  $\rho(S)=+0.603$ .

By the same procedure as in example (i) it was found that

$$\nu_m=0.02817.$$

This is about  $2\frac{1}{2}\%$  less than the value to be expected in the absence of correlation and in fact less than  $(\log 2)/a$ .

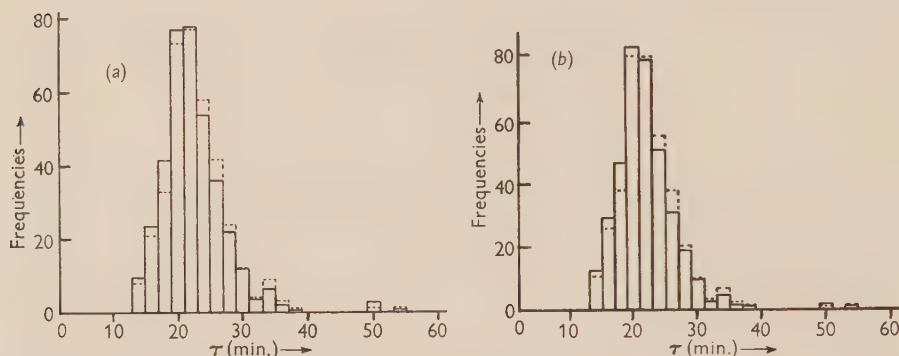


Fig. 6. (a) *Chromobacterium prodigiosum*. Continuous line histogram: population distribution, calculated from generation time distribution and mother-daughter correlation. Dotted: observed generation time distribution. (b) *C. prodigiosum*. Continuous line histogram: carrier distribution, calculated from generation time distribution and mother-daughter correlation. Dotted: carrier distribution expected in the absence of mother-daughter correlation.

These two examples were chosen out of eighteen available because the sample values of  $\rho(H)$  were extreme. Most of the  $\rho(H)$  were much smaller, the average being slightly positive. As might be expected, positive  $\rho(H)$  tends to increase  $\nu_m$  for a given  $f(\tau)$ , negative  $\rho(H)$  to diminish it. But the product-moment coefficient is not really an appropriate numerical measure of the kind of correlation which is effective in making  $\mathcal{P}(\tau)$  differ from  $f(\tau)$ —the first product-moment itself can be zero even if  $h(\tau_1; \tau_2) \neq f(\tau_1)f(\tau_2)$ .

It may be added that (18) (with  $D$  for  $\nu_m$ ,  $f_D(\tau)$  for  $f(\tau)$  etc.) applies equally to continuous cultures.

I conclude that the effect of mother-daughter correlation on growth rate can be neglected for the time being; that equation (10) gives the relation between  $\nu_m$  and  $f(\tau)$  with sufficient accuracy for present needs. However, it cannot yet be said that correlation does not sensibly affect the selective power of the system; we do not know that  $\mathcal{P}(\tau)$  and  $f(\tau)$  are of the same order of magnitude for fairly large  $\tau$ . It is still possible, moreover, that grandmother-granddaughter (or higher order) correlation is not negligible.

## COMMENT

The foregoing paragraphs are mainly intended to provide part of a theoretical background for the detailed study of bacterial growth in continuous cultures, and do not in themselves call for further discussion. It is worth while, however, to consider the probable range of validity of the principal assumptions.

(i) In all actual cultures the number of their proper organisms is so large that the assumption of (mathematical) continuity and determinate behaviour cannot lead to false results. Strictly interpreted, equation (16), for example, is an assertion about expectations only—there is a finite though exceedingly small chance that all the organisms might be washed out of a continuous culture vessel under conditions favourable to continued growth. In fact the culture is self-regulating, and any accidental change in population density is compensated by a changed growth rate which tends to restore the equilibrium. Where the future of the culture turns on the presence of a few organisms (e.g. mutants) only, a deterministic treatment is no longer adequate.

(ii) The steady growth of a batch culture can be maintained for a relatively short time only. However, some calculations by Dr W. A. O'N. Waugh (private communication) show that a few generations after the end of the lag phase are enough to produce an unchanging age distribution in the population. On the other hand, the duration of a continuous culture has no definite limit, and may chance to be prolonged for months (see, for example, Herbert *et al.* 1956). In uncomplicated cases there is then no doubt that a steady state is reached and maintained.

(iii) The assumption of 'perfect' mixing—of instantaneous and homogeneous dispersal of the ingoing medium—greatly simplifies the theoretical and experimental analysis of continuous cultures. It is obvious that for purposes of study, at least, the degree of mixing should be well defined in some sense, but it is not at all obvious that an adequate approximation to 'perfect' mixing is practically attainable. The exigencies of the assumption can be appreciated thus: let a closed surface be imagined to be drawn anywhere within the culture fluid. Then the rate at which fresh medium enters this surface must bear a constant ratio to the volume enclosed by it, no matter what its shape or disposition. A continuous culture is particularly sensitive to lack of homogeneity, because of the steep fall in output near the critical dilution rate. Moreover, the growth rate is not strictly equal to the dilution rate unless the mixing is perfect. This subject needs greater attention than has hitherto been given it, and cannot properly be discussed here. Some experiments of my own, not yet published, suggest that mixing can be made sensibly perfect in laboratory apparatus up to a few litres in capacity.

(iv) Two assumptions commonly made in treating continuous cultures, but not made explicit above are: (a) that growth rate responds instantly to changes in medium concentration; (b) that growth is not inhibited or promoted by products of metabolism or by imbalance in the medium caused by the organisms themselves. Failure of either assumption can demonstrably lead to sustained oscillations in population density.

## SYNOPSIS

Here the principal results are summarized without reference to the limitations imposed by the assumptions made.

(i) The growth rate ( $\nu$ ) of a culture is not simply related to the generation time distribution. In batch cultures, when  $\nu$  has nearly its maximum value  $\nu_m$ , the relation is given by equation (10), where  $f(\tau)$  is the frequency function of generation time. In continuous cultures  $\nu$  is equal to the dilution rate  $D$ . Since  $D < \nu_m$ , growth is restricted; the frequency function must alter, in scale if not in shape, so as to satisfy equation (16).

(ii) The frequency function of age ( $\phi(a)$ ) is always a J-shaped curve with a finite maximum at  $a=0$ ; the younger organisms are present in the greater numbers. Its dependence on the generation time distribution is given by equations (9) and (15).

(iii) It is convenient to distinguish two different generation time distributions in a growing culture. The population distribution,  $\mathcal{P}(\tau)$ , is the distribution for all organisms present at any one instant. When there is negligible correlation between the generation times of related organisms,  $\mathcal{P}(\tau) \equiv f(\tau)$ . The mothers of the organisms present at any one time have a different generation time distribution, the carrier distribution,  $\mathcal{C}(\tau)$ . In continuous cultures, some of the organisms present at any one time will be washed out before they divide. Those which remain in the vessel until their termination have the distribution  $\mathcal{C}(\tau)$ . Equations (11) and (17) determine  $\mathcal{C}(\tau)$  in terms of  $f(\tau)$ .

(iv) Correlation between the generation times of sister organisms does not affect the growth rate. Positive mother-daughter correlation increases it, negative diminishes it. The population distribution depends on the joint distribution of generation times of mothers and daughters according to equation (18), and is not then equal to  $f(\tau)$  in general. The available data suggest that mother-daughter correlation is small enough to be neglected for the time being. If it has to be taken into account,  $f(\tau)$  must be replaced by  $\mathcal{P}(\tau)$  (as determined by (18)) in expressions for  $\nu$ ,  $\phi(a)$  and  $\mathcal{C}(\tau)$ .

I am indebted to Dr D. Herbert for some suggestions and criticism.

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## Liberation and Osmotic Properties of the Protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea*

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**SUMMARY:** Stable protoplasts may be released from *Micrococcus lysodeikticus* and *Sarcina lutea* by digestion of the cell-wall with lysozyme in sucrose or NaCl solutions having an osmotic pressure of some 25 atmospheres, but not in glycerol solutions of the same osmotic pressure. The stability of the protoplasts depends not only upon the depression of the water activity by the solute but upon an osmotic pressure exerted against the protoplast (plasma-) membrane. The permeability of the protoplast membrane to a number of solutes resembles that of the osmotic barrier of intact *Staphylococcus aureus*.

This paper describes work on the protoplasts of *Micrococcus lysodeikticus* and *Sarcina lutea* which was completed just over a year ago in the Department of Biochemistry, University of Cambridge. It was undertaken as part of a research programme designed to investigate the physiological role of the plasma-membrane in bacteria (Mitchell & Moyle, 1956*a, b*).

Three years ago, Weibull (1953) discovered that spherical bodies, which were thought to be intact protoplasts, emerged from *Bacillus megaterium* when the cell walls were digested by lysozyme in solutions of which the water activity had been depressed by the addition of polyethylene glycol, sucrose or certain other solutes. It was not known, however, whether the mechanism of the stabilizing action of the solutes was by the exertion of an osmotic pressure across the membrane, thus preventing osmotic explosion of the protoplast, or whether the solutes prevented the swelling of hydrated constituents of the protoplasm which would otherwise have ruptured the external membrane. It was therefore of interest to measure the permeability of isolated protoplasts to a range of solutes; for it would be expected that if the protoplasts were stabilized by the external osmotic pressure, only non-penetrating solutes would be effective as stabilizers, whereas if the mechanism of stabilization were by dehydration of components of the cytoplasm the effectiveness of a solute would depend only upon the lowering of the activity of the water. The lysozyme-sensitive cocci were chosen for study because we wished to obtain data which might usefully be compared with similar data on the internal osmotic pressure and permeability of *Staphylococcus aureus*.

Grula & Hartsell (1954) attempted to prepare protoplasts of *Micrococcus lysodeikticus* by the method used for *Bacillus megaterium* (Weibull, 1953), but most of the protoplasts swelled and disintegrated as soon as they were released from the cell-walls. Dr M. R. J. Salton (personal communication) attempted to obtain protoplasts from *M. lysodeikticus* in higher sucrose concentrations, but could observe no indication of protoplast formation from the changes in light-

scattering of the suspensions in sucrose. In view of our observations on *Staphylococcus aureus* which implied that the osmotic pressure was between 20 and 25 atmospheres (Mitchell & Moyle, 1956*a*), it was possible that the protoplasts of the cocci might require at least M-sucrose or M-saline solutions to preserve them from osmotic explosion. Moreover, it seemed possible that at very high sucrose concentrations, the depression of the light scattering which would result from the elevation of the refractive index of the medium might be sufficient to make the protoplasts invisible by light scattering unless they were subsequently transferred to a medium of lower refractive index.

## METHODS

*Growth and preparation of organisms.* Cultures of *Micrococcus lysodeikticus* (NCTC 2665) and *Sarcina lutea* (laboratory strain) were grown aerobically at 25° in a medium containing 3 % (w/v) tryptic digest of casein, 1 % glucose and 0.1 % Marmite, using the rotated flask technique (Mitchell, 1949). The organisms were harvested at a concentration equivalent to 1.5–2 mg. dry weight/ml., washed twice with distilled water (for *M. lysodeikticus*) or 0.5 M-NaCl (for *S. lutea*) and suspended at a concentration corresponding to c. 100 mg. dry weight/ml. in distilled water (for *M. lysodeikticus*) or 0.5 M-NaCl (for *S. lutea*).

*Measurement of turbidity.* The extinction of 1 cm. depth of suspension was measured at a wavelength of 700 m $\mu$ ., using the Beckman model DU spectrophotometer.

*Microscopy.* The intact organisms and protoplasts were examined with a Cooke Troughton and Simms binocular phase-contrast microscope in films of aqueous solution sealed between slide and coverslip with vaseline.

### *Protoplast release and stability*

*Method I. Visibility of protoplasts and intact organisms in NaCl and sucrose solutions.* Samples of organisms were incubated for 2 hr. at 25° at a standard concentration corresponding to 200  $\mu$ g. dry wt. organisms/ml., with a range of concentrations of NaCl or sucrose in 0.04 M-sodium phosphate buffer (pH 6.8), containing 10  $\mu$ g. (for *Micrococcus lysodeikticus*) or 40  $\mu$ g. (for *Sarcina lutea*) crystalline egg-white lysozyme/ml. The turbidities of the suspensions were then measured and compared with those of controls in which the lysozyme was omitted.

*Method II. Dependence of protoplast stability on osmotic pressure of release-medium.* Samples of suspensions were incubated as above but at a concentration corresponding to 10 mg. dry wt. organisms/ml. Samples (0.1 ml.) of the incubated suspensions were pipetted into 5 ml. samples of M-NaCl containing 0.01 M-sodium phosphate buffer at pH 6.8. After 20 min. at 20°, the turbidities of the suspensions were measured. Controls were done omitting lysozyme only.

*Method III. Dependence of protoplast stability on osmotic pressure of NaCl solutions to which protoplasts are transferred after release in NaCl or sucrose.* The protoplasts were released by incubating the organisms with lysozyme at 25° for 2 hr. at a concentration corresponding to 10 mg. dry wt. organisms/ml.



in 1.2 M-sucrose or 2 M-NaCl containing phosphate buffer as in (I). Samples (0.1 ml.) of the protoplast suspension were pipetted into 5 ml. samples of NaCl ranging from 0 to 2 M, containing 0.01 M-sodium phosphate buffer at pH 6.8. After 20 min. the turbidities of the suspensions were measured. Controls were done omitting lysozyme only.

*Method IV. Dependence of protoplast stability on osmotic pressure of sucrose, NaCl or glycerol solutions to which protoplasts are transferred after release in 1.2 M-sucrose.* The protoplasts were released in 1.2 M-sucrose as in method III. The incubated suspension was mixed with 5 vol. 1.2 M-NaCl (to decrease the specific gravity) and centrifuged at 20°; and the protoplasts were redispersed in 1.2 M-NaCl to give a suspension density corresponding to 100 mg. original dry wt. organisms/ml. Samples (0.5 ml.) of the protoplast suspension were pipetted into 4.5 ml. samples of NaCl, sucrose or glycerol arranged to give final concentrations ranging from 0 to 2 M in 0.01 M-sodium phosphate buffer at pH 6.8. After 30 min. incubation at 25°, samples (0.1 ml.) of the protoplast suspensions in the NaCl, sucrose and glycerol solutions were pipetted into 5 ml. samples of 1.2 M-NaCl containing 0.01 M-sodium phosphate buffer at pH 6.8, and after a further 20 min. the turbidities were measured.

*Method V. Determination of protoplast permeability.* The protoplasts were released in sucrose as in method III. The incubated suspension was mixed with 5 vol. 1.2 M-NaCl and centrifuged at 20°; and the protoplasts were redispersed in 1.2 M-NaCl to give a suspension density corresponding to 10 mg. original dry weight organisms/ml. Samples (0.1 ml.) of the protoplast suspension were pipetted into 1.5 molal solutions (in 0.01 M-sodium phosphate buffer at pH 6.8) of the solutes to which the permeability was to be measured. The rate of lysis of the protoplasts, measured by the change of light scattering, was taken as an approximate index of the rate of permeation of the solute.

## RESULTS

### *'Protoplast' release*

The effect of lysozyme treatment (Method I) on the turbidity of suspensions of *Micrococcus lysodeikticus* in concentrations of sucrose from 0.1 to 1.0 M is shown in Fig. 1. At the low sucrose concentrations the lysozyme-treated suspensions scattered very little light, but, although the amount of light scattered was still small, at concentrations of sucrose between 0.5 and 1.0 M they scattered between 10 and 20 % as much light as the corresponding untreated suspensions. This suggests that protoplasts were being liberated and were stable in sucrose concentrations approaching 1 M, but that their light scattering (like that of the intact organisms) was depressed by the high refractive index of the sucrose solutions. The lysozyme treatment was therefore done in NaCl solutions (method I) with the results shown in Fig. 2. At concentrations of NaCl above 0.5 M the lysozyme-treated organisms scattered between 50 and 60 % as much light as the untreated organisms, but below 0.5 M-NaCl the light scattered by the lysozyme-treated organisms fell towards some 5 % of that scattered by the untreated organisms. Similar results were



obtained with *Sarcina lutea*, the only significant difference being that *S. lutea* tended to lyse slowly in distilled water. The suspensions of *S. lutea* were therefore washed and made up in 0.5 M-NaCl in place of distilled water.

Phase-contrast microscopy showed that the lysozyme-treated suspensions of both organisms in concentrations of NaCl above 1.0 M contained spherical bodies of approximately the same diameter but of lower contrast than organisms of corresponding control suspensions, and whereas the organisms of the controls adhered to one another the spherical bodies in the lysozyme-treated suspensions were separate. In concentrations of NaCl below 1.0 M, the lysozyme-treated suspensions contained spherical bodies (which were larger than the cells of corresponding control suspensions) together with ghost membranes of extremely low contrast. The lower the NaCl concentration, the larger

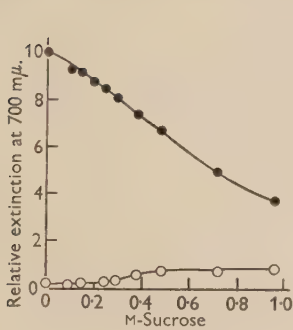


Fig. 1

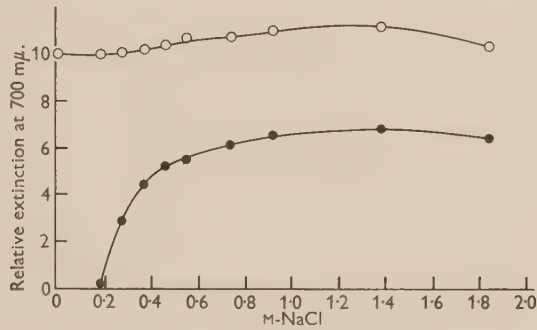


Fig. 2

Fig. 1. The dependence of the extinction of suspensions of *Micrococcus lysodeikticus* on the concentration of sucrose in the suspension medium after incubation with lysozyme (○) and without lysozyme (●) (see method I).

Fig. 2. The dependence of the extinction of suspensions of *Micrococcus lysodeikticus* on the concentration of NaCl in the suspension medium after incubation with lysozyme (●) and without lysozyme (○) (see method I).

was the ratio of ghosts to intact spherical bodies; and no intermediate forms were present. It was evident that the spherical bodies corresponded to the light-scattering units of the suspensions and that since they either remained intact or lysed completely, the amount of light scattered by a suspension was approximately proportional to the number of surviving spherical bodies. Microscopic examination of the suspensions treated with lysozyme in sucrose (corresponding to Fig. 1) was not successful because the contrast was much decreased by the high refractive index of the sucrose solutions; but by diluting the sucrose-containing suspensions with M-NaCl, the same general pattern as described above could be observed.

In order to ascertain quantitatively whether lysozyme treatment in NaCl solutions followed the same pattern as in sucrose solutions, samples of organisms were treated with lysozyme in a range of sucrose and NaCl concentrations, and subsequently transferred to M-NaCl to measure the turbidity (method II). Fig. 3 shows the results of such an experiment with *Micrococcus lysodeikticus*, harvested at a population density corresponding to c. 1.5 mg. dry weight/ml.;

similar results were obtained with *Sarcina lutea*. It is evident that to stabilize 50 % of the light-scattering units of the lysozyme-treated suspension requires the presence of 0.33 M-NaCl or 0.53 M-sucrose. From the vapour-pressure equilibrium data of Robinson & Sinclair (1934), it is found that 0.53 M-sucrose corresponds to 0.34 M-NaCl in water activity (or osmotic pressure for a membrane impermeable to sucrose and NaCl). Thus, the stabilization of the lysozyme-treated suspensions is practically the same whether the water activity is depressed (or osmotic pressure raised) by NaCl or by sucrose.

The stabilization of the lysozyme-treated organisms in the higher NaCl and sucrose concentrations might have been due either to stabilization of protoplasts which had been completely released from the cell walls, or to an inhibitory effect of the sucrose and NaCl on the lysozyme reaction. The latter

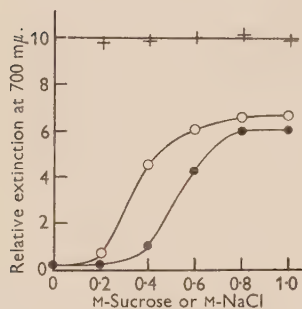


Fig. 3

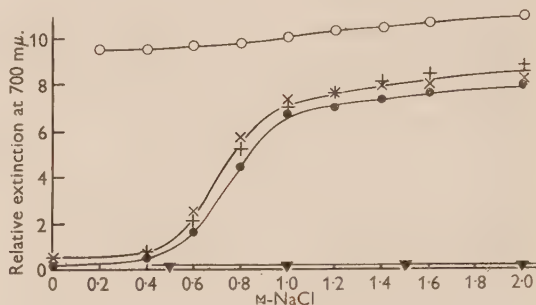


Fig. 4

Fig. 3. The dependence of the extinction of suspensions of *Micrococcus lysodeikticus* in M-NaCl on the concentration of NaCl or sucrose in which the cells were previously incubated with lysozyme (NaCl, O; sucrose, ●) and without lysozyme (mean for NaCl and sucrose +) (see method II).

Fig. 4. The dependence of the extinction of suspensions of *Sarcina lutea* on the concentration of NaCl to which the cells are transferred after incubation in 2M-glycerol, 1.2M-sucrose, or M or 2M-NaCl solutions with lysozyme (2M-glycerol, ▼; 1.2M-sucrose, ●; M-NaCl, x; 2M-NaCl, +) and without lysozyme (mean for NaCl, sucrose and glycerol, O) (see method III).

possibility was eliminated by showing that the organisms in the suspensions treated with lysozyme at high sucrose or NaCl concentrations lysed when transferred to solutions of lower concentration. The organisms were treated with lysozyme in 1.2 M-sucrose, or M or 2 M-NaCl solutions and subsequently transferred to a range of NaCl concentrations (method III). Fig. 4 shows such an experiment with *Sarcina lutea*. The turbidity of the suspensions that were treated with lysozyme in M or 2M-NaCl was slightly higher than those treated with lysozyme in 1.2 M-sucrose over the whole range of NaCl concentrations to which they were transferred. However, the dependence of the stability of the light-scattering elements upon the NaCl concentration to which the suspensions were transferred was practically the same whether lysozyme treatment was done in 1.2 M-sucrose, M or 2 M-NaCl. It followed that the action of the high concentrations of sucrose or NaCl was not due to an inhibition of the lysozyme reaction, but could be attributed to stabilization of

'protoplasts' which had been completely released from the normal mechanical protection of the cell wall.

In order to determine whether the 'protoplasts' were stabilized by an osmotic pressure acting across a semi-permeable membrane, or whether the stabilization was due to dehydration of protoplasmic constituents which would otherwise swell and cause the 'protoplasts' to disintegrate, the lysozyme treatment was carried out in 2 M-glycerol in place of sucrose (method III); for it was shown by Fischer (1903) that many bacteria are permeable to glycerol. The results (Fig. 4), showed that the 'protoplasts' were not stabilized when released in 2 M-glycerol. The stabilization cannot therefore be due to lowering of the water activity alone: it must be caused by the substitution of an osmotic

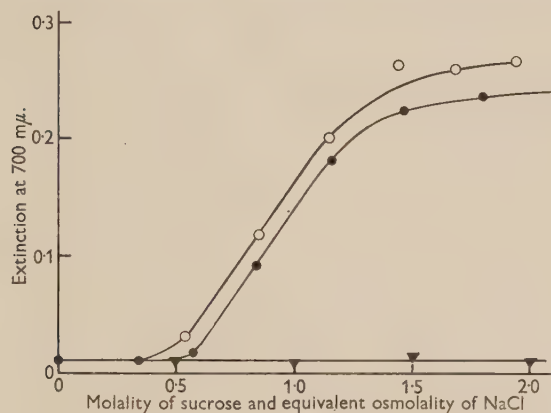


Fig. 5

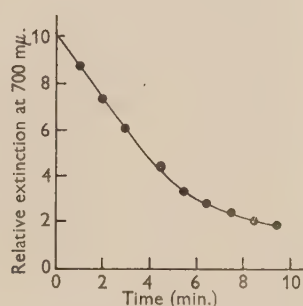


Fig. 6

Fig. 5. The dependence of the extinction of suspensions of 'protoplasts' of *Micrococcus lysodeikticus* in 1.2 M-NaCl on the concentration of glycerol (▼), sucrose (●) or NaCl (○) to which they are intermediately transferred after release by lysozyme in 1.2 M-sucrose (see method IV).

Fig. 6. The dependence of the extinction of 'protoplasts' of *Micrococcus lysodeikticus* suspended in 1.5 molal D-ribose on time at 20° (see method V).

pressure at the outer surface of the 'protoplast' membrane for the hydrostatic pressure exerted by the cell-wall of the intact organism. The molarity of NaCl at which half the 'protoplasts' of *Sarcina lutea* are stabilized is *c.* 0.7 M in the experiment of Fig. 4: it corresponds to an osmotic pressure of between 25 and 30 atmospheres. This probably represents about the mean osmotic pressure of the cells.

An alternative and perhaps preferable method of examining the dependence of the stability of the 'protoplasts' on the osmotic pressure and composition of the suspension medium is to release a large batch of 'protoplasts' in 1.2 M-sucrose, to transfer samples of these 'protoplasts' to ranges of concentration of NaCl, sucrose and glycerol, and then after a fixed time interval to determine the proportion of surviving 'protoplasts' by measuring their light-scattering when transferred to 1.2 M-NaCl (method IV). The results of such an experiment with *Micrococcus lysodeikticus* are plotted in Fig. 5. The scale of concentration has been converted to molality of sucrose and equivalent osmolality of NaCl by

means of the vapour pressure equilibrium data of Robinson & Sinclair (1934) and specific gravity tables. As with *Sarcina lutea*, sucrose and NaCl are about equally effective in stabilizing the 'protoplasts' from *M. lysodeikticus* while glycerol is not effective. The osmolality required to stabilize half the 'protoplasts' is *c.* 0.9, corresponding to an osmotic pressure of *c.* 20 atmospheres. The osmolality of sucrose or NaCl solutions required to stabilize half the 'protoplasts' of *M. lysodeikticus* and *S. lutea* varied from one culture to another about a mean of 1.0 osmolal, when harvested at a dry weight of 2.0 mg./ml.

#### 'Protoplast' permeability

The rate of lysis of the 'protoplasts' of *Micrococcus lysodeikticus* and *Sarcina lutea* was less than 10%/hr. at 20° in 1.5 molal solutions of the following solutes in 0.01 M-sodium phosphate buffer at pH 6.8 (except where otherwise stated): NaCl, KCl, NH<sub>4</sub>Cl, MgCl<sub>2</sub>, NaBr, KBr, Na-acetate (pH 9), K-acetate (pH 9), K<sub>2</sub>SO<sub>4</sub>, (KH<sub>2</sub>PO<sub>4</sub> + K<sub>2</sub>HPO<sub>4</sub>), Na-glutamate, lysine-HCl, D-glucose, D-fructose, D-mannose, D-galactose, D-sorbose and sucrose (method V). Since it was possible that metabolism of the carbohydrates might affect the stability of the 'protoplasts', the effect of the presence of 10<sup>-2</sup> M-KCN, 10<sup>-2</sup> M-sodium iodoacetate, 10<sup>-3</sup> M-sodium dinitrophenate or 10<sup>-5</sup> M-HgCl<sub>2</sub> on the rate of lysis of the 'protoplasts' was determined. The rate of lysis was, as in the case of the untreated suspensions, less than 10%/hr. The 'protoplast' membrane is therefore only slightly permeable to the above solutes under the conditions of these experiments. However, under the same conditions as above, the 'protoplasts' were *c.* 70 % lysed in 3 sec. in glycerol, 20 sec. in erythritol, 5 min. in D-ribose, 30 min. in L-arabinose and 1 hr. in D-sorbitol. The progress of lysis of 'protoplasts' of *Micrococcus lysodeikticus* in D-ribose is shown in Fig. 6.

It is not possible to estimate accurately the rate of equilibration of the solutes across the 'protoplast' membrane from measurements such as those of Fig. 6 without much additional information. However, an approximate estimate of the permeability of the membrane can be obtained since we know from the data of Figs. 4 and 5 that 70 % lysis of the 'protoplasts' corresponds to about an osmotic pressure difference of 0.75 osmolar across the 'protoplast' membrane, or to half equilibration of the 1.5 molal non-electrolyte solutions used in these experiments. Thus, we may say that the 70 % lysis times given above correspond approximately to the times for half equilibration of the solutes across the 'protoplast' membrane.

#### DISCUSSION

There is no doubt that the 'protoplasts' liberated from *Micrococcus lysodeikticus* and *Sarcina lutea* by lysozyme possess the properties that would be expected of the protoplasm of the cell covered by a plasma membrane. It is, however, important to emphasize that we do not yet know whether the composition of the membrane of the 'protoplasts' liberated by lysozyme is the



same as that of the plasma-membrane of the intact organism. Even so, we suggest that the removal of the inverted commas from the word protoplast when used to describe the bodies which are liberated from *M. lysodeikticus* and *S. lutea* by lysozyme in media of high osmotic pressure is justified on the following grounds: (i) Lysozyme is known to dissolve the cell wall of these organisms (Salton, 1954). (ii) The bodies are approximately the same size as the intact organisms, and of only slightly lower contrast in phase-contrast microscopy, when suspended in media in which the degree of lysis is small. (iii) The bodies scatter about two-thirds as much light as the intact organisms. (iv) The bodies are covered by a semipermeable membrane which retains the small molecular weight internal solutes of which the total osmotic pressure is some 20 atmospheres, and the bodies are osmotically exploded in media of low osmotic pressure. (v) After lysis in media of low osmotic pressure the empty membranes are visible by phase-contrast microscopy. (vi) In media containing a high concentration of solutes known to be unable to penetrate the plasma membrane of *Staphylococcus aureus* and *Escherichia coli* (Mitchell & Moyle, 1956*a*), the membrane of the bodies is subject to an external osmotic pressure which substitutes for the hydrostatic pressure normally exerted by the cell wall and prevents explosion of the bodies. (vii) When the osmotic pressure of the external medium is raised by glycerol or other solutes which are known to penetrate the plasma membrane of intact bacteria, the passage of the solute through the 'protoplast' membrane results in osmotic explosion.

Since this work was completed, Weibull (1955) has described experiments on the osmotic properties of the protoplasts of *Bacillus megaterium* with which our observations are essentially in agreement. He observed, however, that the total volume of the protoplasts of *B. megaterium* was independent of the osmotic pressure of the sucrose solution in which they were released, over a range from 0.125 to 0.4 M. In the work described here, there was no indication that the protoplasts of the cocci distinguished between the osmotic pressure of the medium in which they were released and that to which they were subsequently transferred. It may, perhaps, be relevant that whereas the protoplasts of *B. megaterium* must change shape from cylinders to spheres, there is little or no change of shape of the protoplasts of the cocci during release by lysozyme.

The aim of the work described in the present paper was to obtain a general view of the effectiveness of the membrane of *Micrococcus lysodeikticus* and *Sarcina lutea* as a barrier to the free diffusion of solutes between the cell interior and the environment. It is, perhaps, relevant to point out that the membrane does not act only as an osmotic barrier. Its most specific function is to link the internal and external media, allowing entry of specific metabolites and exit of end-products of metabolism. The rate of lysis of the protoplasts in 1.5 molal glucose and in the other hexose solutions shows that these sugars enter both the normal (semi-anaerobic) and the inhibitor-treated protoplasts at a rate of less than 0.1 mole/l. protoplast volume/hr. On the other hand, we have recently found that under aerobic conditions the intact cocci or the protoplasts oxidize glucose at a rate corresponding to 1.0 mole or more/l.

protoplast volume/hr. It follows that the movement of glucose through the plasma membrane is closely linked to its oxidation. The implication of this coupling has been discussed briefly elsewhere (Mitchell & Moyle, 1956*b*), and we have suggested that glucose is carried through the membrane on an enzyme or on a protein carrier of equivalent specificity and affinity. Further work on this problem, which is now in progress, will be described shortly in another paper.

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## A Study of the Cider-sickness Bacillus—a New Variety of *Zymomonas anaerobia*

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**SUMMARY:** Cider sickness is a disorder of sweet low-acid ciders and perries. The causal organism was isolated after anaerobic incubation of the centrifuged deposit from infected cider on a medium which contained apple juice, 1 % (w/v) yeast extract and 10 µg. Actidione/ml., at pH 4.5. The causal organism found was a Gram-negative motile rod for which the name *Zymomonas anaerobia* var. *pomaceae* is proposed on the basis of a comparison of its morphology, cultural and biochemical characters with those of *Termobacterium mobile* Lindner (synonyms: *Pseudomonas lindneri*, *Zymomonas mobile*) and *Achromobacter anaerobium* Shimwell (synonym: *Saccharomonas anaerobia*). It is proposed that the generic name *Zymomonas*, Kluyver & van Niel (1936) be adopted for the organisms isolated by Lindner (1928) and Shimwell (1937) and the organism described in this paper. The distinctive character which places these three organisms together in a separate genus in the tribe Pseudomonadeae is their ability to ferment glucose to give almost a theoretical yield of ethanol.

Sweet low-acid ciders and perries (less than 0.5 g. malic acid/100 ml.) are subject to a disorder called cider sickness. In the early stages of the disorder a thin haze develops, then copious gas production follows as sugar is fermented to ethanol and carbon dioxide. A full fruity aroma develops due to the liberation of acetaldehyde. Later the aroma and flavour become very harsh and a dense precipitate of an aldehyde-tannin complex forms. Cider so spoiled never regains its former quality, although after storage for 6 months it may be used in blending. The disorder was recognized in England in 1903 (Lloyd), and although Barker & Hillier (1912) isolated an organism from sick cider (which they called the cider-sickness bacillus) their description of it is incomplete by modern standards and they made no attempt to classify it. It seems probable, however, that their isolate and those made by Millis (1951) are identical. Grove (1914, 1916) and Barker (1948) described various aspects of the disorder and practical measures to combat it. In France a similar problem exists. Warcollier (1928) recorded the condition under a number of names, *la tourne* and *la pousse*. Unfortunately these names were given to disorders caused by lactobacilli and the confusion has been perpetuated in Charley's translation (1949). However, the name *framboisé*, now apparently in current use in France, was applied to the disorder by Warcollier (1939), who isolated a mobile anaerobic cocco-bacillus from ciders suffering from this disorder. But later Guittonneau, Mocquot & Tavernier (1940) attributed the development of *framboisé* in cider to the symbiotic growth of yeast and acetic acid bacteria. They claimed that in *framboisé* cider a delicate balance between yeast and

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acetic acid bacteria existed which allowed the oxidation of ethanol by the bacteria to proceed as far as acetaldehyde. This occurred only when the balance was correct and the concentration of oxygen was limiting. They claimed that the accumulated acetaldehyde was responsible for the fruity aroma and the copious precipitate associated with this disorder. Auclair (1955) and Barret (1955) also studied *framboisé* cider. Their results indicated that this disorder, whose outward characters appeared identical with those of cider sickness, may be caused by an organism similar to, if not identical with, the cider-sickness bacillus.

The present study describes the characters of the cider-sickness bacillus and shows its relationship to the organisms *Termobacterium mobile* Lindner (1928) (*Pseudomonas lindneri*; *Zymomonas mobile*) and *Achromobacter anaerobium* Shimwell (1937) (*Saccharomonas anaerobia*).

## METHODS

*Organisms.* Two strains, 1 and 2, of the cider-sickness bacillus were isolated from ciders and perries examined at the Research Station, Long Ashton, Bristol. *Achromobacter anaerobium* NCIB 8227 (*Saccharomonas anaerobia*) was obtained from Mr H. J. Bunker, London. Three strains of *Termobacterium mobile* (*Pseudomonas lindneri*, *Zymomonas mobile*) were obtained from the late Prof. A. J. Kluyver, Delft. Strain L192 was the original received from Lindner 25 years ago and strains 2 and 3 were isolated in Java.

## Media

*Isolation medium.* Apple juice was treated overnight with 1 g. Pectozyme (Messrs Norman, Evans and Rais Ltd., Dudley Road, Manchester) per 100 ml., filtered and 1 % Difco yeast extract added. The pH value was adjusted to 4.5 and the medium filtered again. Agar to 3 % (w/v) was added to make a solid medium. The medium was autoclaved at 7 lb./sq.in. for 15 min. On storage for c. 4 weeks, the liquid medium develops a fine dark precipitate which makes it unsatisfactory for some purposes, although growth is in no way inhibited.

*Stock culture medium.* Cultures were maintained as stabs in screw-cap bottles containing apple juice diluted 1 part + 3 of water, + 1 % (w/v) Difco yeast extract, solidified with 10 % (w/v) gelatin at pH 5.5. They remained viable in this medium for at least 17 weeks.

*Carbohydrate fermentation tests.* Seitz-filtered carbohydrate solutions were added to sterile 1 % Difco yeast extract at pH 5.5, to give a final concentration of 1 % carbohydrate. Bromocresol green was used as indicator.

*Indole production.* 1 % Difco yeast extract containing 2 mg. DL-tryptophan/ml. at pH 5.5 was used. The indole reagent was made as recommended by Mackie & McCartney (1946). The test was performed after incubation for 10 days.

*Nitrate reduction.* 1 % Difco yeast extract, 0.4 % NaNO<sub>3</sub>, 1 % glucose, pH 5.5;  $\alpha$ -naphthylamine and sulphanilic acid solutions were used for the detection of nitrite (Topley & Wilson, 1946). The test was performed after incubation for 10 days.



*Ammonia production.* 1 % Difco yeast extract + 1 % glucose (adjusted to pH 5.5). After incubation for 10 days, Nessler's reagent was used to test for the presence of ammonia.

*Hydrogen sulphide production.* The same medium was used as that for detecting ammonia production. Lead acetate paper suspended from the plug was used to detect hydrogen sulphide.

*Methyl red and Voges-Proskauer tests.* The medium contained 0.5 % glucose + 0.5 %  $K_2HPO_4$  + 0.5 % Difco yeast extract + 0.5 % Difco peptone; the pH value was adjusted to 6.5. O'Meara's modification (Mackie & McCartney, 1946) was used to detect the presence of acetylmethylcarbinol after incubation for 10 days. Methyl red solution was made as recommended in Mackie & McCartney (1946); the test being performed after incubation for 10 days.

*Temperature.* All cultures were incubated at 25° unless otherwise stated.

*Isolation procedure.* The isolation of spoilage bacteria from cider in the presence of large amounts of yeast presents considerable difficulty. By adjusting the pH value of the apple juice medium to 4.5, and using an anaerobic atmosphere, bacteria whose true habitat is not cider are discouraged, but these conditions allow both yeasts and cider bacteria to grow.

Attempts to separate yeast and bacteria by differential centrifugation or filtration were unsuccessful. Better results were obtained by adding to the isolation medium substances which inhibited the growth of yeasts but not of bacteria. Green & Gray (1950) found Actidione (The Upjohn Co., Kalamazoo, Michigan, U.S.A.) helpful in the isolation of bacteria from brewery materials, and at 10  $\mu$ g./ml. in apple juice medium this compound suppressed the growth of most cider yeasts whilst allowing lactobacilli, acetic acid bacteria and the cider-sickness bacillus to grow. Malachite green in apple juice medium (250  $\mu$ g./ml.) retarded the growth of yeasts and inhibited lactobacilli completely. Since it had little effect on the cider-sickness bacillus or on acetic acid bacteria, it was used for their isolation. Actidione was used, however, for all routine investigations since it allowed all the common cider-spoilage bacteria to grow. Phillips & Hanel (1950) claimed that moulds were inhibited by Actidione, but this did not apply under the conditions of these experiments. The procedure finally adopted for the isolation of bacteria in cider was to centrifuge a 10 ml. sample of cider, plate the deposit on apple juice medium containing 10  $\mu$ g. Actidione/ml., and incubate the plate anaerobically for 4-5 days.

## RESULTS

The sickness organism, whose properties are described in detail later, was isolated from the thirty naturally sweet sick ciders or perries examined. When inoculated into pasteurized cider or apple juice these isolates all produced a vigorous ethanolic fermentation with an aroma and flavour typical of the original disorder, as judged by a panel of experienced cider-tasters. Two strains were found to differ only in morphological appearance; these have been called strains 1 and 2.

*Morphology.* Strain 1 showed short rods mainly occurring as diplobacilli,

3.5–4.0  $\mu$ . long and 1.5  $\mu$ . wide, but also singly 2.0  $\mu$ . long and 1.5  $\mu$ . wide. Gram-negative, staining evenly. No spores or capsules detected. When grown in unfavourable conditions or when old, the organisms became pleomorphic. Actively motile in young cultures in glucose yeast-extract medium, but frequently non-motile in apple juice and cider.

Strain 2 was like strain 1 in shape, size and motility of the individual organisms. In addition, this strain formed rosettes or clumps of organisms which, in young cultures, swarmed over each other; whole clumps moved about in a hanging-drop preparation.

*Cultural characters.* With strain 1 on apple-juice agar medium under anaerobic incubation, the colonies grew to 0.5 mm. diam. in 2 days. At 7–8 days the colonies had reached maximum size, 3–5 mm. diam. The colonies were circular, regular, entire edged, low convex, butyrous, opaque and of pale buff after 5 days. A sickly sweet aroma, quite characteristic, was produced and persisted for about a week. In apple-juice liquid medium during the first 2–3 days there was dense and even turbidity, but as fermentation ceased, the supernatant fluid cleared and a creamy compact deposit formed which darkened with age as a tannin-aldehyde complex settled out.

With strain 2, under the same conditions, the organism produced colonies which were of high-convex, dewdrop appearance, and which were less opaque than strain 1. At 8–10 days, the colonies showed denser patches in a watery buff-coloured background; at the same time they flattened and spread so that colonies which were discrete at 4 days gradually merged to produce a solid streak of growth. In apple-juice liquid medium, the clumps of organisms seen microscopically caused a dense granular turbidity when fermentation was active. On standing a week, the supernatant fluid became clear, with a flocculent deposit which was cream at first but later darkened.

*Biochemical and physiological characters.* The results of tests on the cider-sickness bacillus, *Termobacterium mobile* and *Achromobacter anaerobium*, are shown in Table 1.

*Glucose fermentation.* Both strains of the cider-sickness bacillus gave almost theoretical conversion of glucose to ethanol (see Table 2). The mechanism of this fermentation has not been studied for the cider-sickness bacillus, but Gibbs & DeMoss (1951, 1954) and DeMoss (1953) established that whilst the products of glucose fermentation by *Termobacterium mobile* (*Pseudomonas lindneri*) are similar to those of a yeast fermentation, the mechanism is different. They found that carbon dioxide was formed from C<sub>1</sub> and C<sub>4</sub> of glucose, whilst the methyl group of ethanol was derived from C<sub>3</sub> and C<sub>6</sub> of glucose. Gunsalus, Horecker & Wood (1955) cited unpublished data of Roa & Gunsalus that *T. mobile* does, however, possess a yeast-like carboxylase.

*Sulphur dioxide tolerance.* Since sulphur dioxide is a permissible preservative in English cider it was of practical importance to test the tolerance of the organism to it. Sulphur dioxide was added to apple juice medium at 100, 200, 300, 400, 500, 750, 1000 and 2000  $\mu$ g./ml. Growth was delayed 8 days at 500  $\mu$ g./ml.; concentrations above 750  $\mu$ g./ml. inhibited growth completely. There was no inhibition, however, at the legally permissible concentration of

Table 1. Comparison of *cider-sickness bacillus* with *Termobacterium mobile* and *Achromobacter anaerobium*

	Cider-sickness bacillus	Growth in fluid medium	pH range	Optimum tem- perature (° C.)	Motility	Acetylmethyl- carbinol production	Methyl-red test	Nitrite production	Indole production	Catalase production	H <sub>2</sub> S production	Gelatin liquefaction	Sorbitol	Dulcitol	Mannitol	Raffinose	Rhamnose	Arabinose	Xylose	Sucrose	Maltose	Fructose	Lactose	Galactose	Glucose		
	(a) Strain 1	Dense even turbidity, later formed a compact deposit	3.5 7.9	30	+					+	+		+								G				G		
	(b) Strain 2	Dense granular turbidity, later formed a diffuse deposit	3.5 7.9	30	+					+	+										G				G		
	<i>Achromobacter anaerobium</i>	Dense slightly granular turbidity, later formed a semi-flocculent deposit	3.4 7.5	30*	+					+	+		+								G				G		
	<i>Termobacterium mobile</i>	Dense granular turbidity, later formed a flocculent deposit	0	30*	-					+	+		+			+				G	-	G	-	-	G		
	(a) L.192	Dense even turbidity, later formed a compact deposit	0	30*	+					+	+		+							G	-	G	-	-	G		
	(b) Strain 2	Dense granular turbidity, later formed a diffuse flocculent deposit	0	30*	+					+	+		+							G	-	G	-	-	G		
	(c) Strain 3	Dense granular turbidity, later formed a diffuse flocculent deposit	0	30*	+					+	+		+							G	-	G	-	-	G		

Note. G = gas production and growth; ± = growth sometimes occurred but no gas production; - = no growth or negative test; \* = as quoted in literature; 0 = information not available.

200  $\mu\text{g.}/\text{ml.}$  It seems probable that aldehyde liberated by the sickness bacillus combines rapidly with the sulphur dioxide to render it inactive.

*Tolerance to various pH values.* Grove (1914) observed that low-acid sweet ciders were particularly susceptible to cider sickness; the present studies have shown that the pH value of a cider, rather than its titratable acidity, controls the growth of the causative organism. Samples of apple-juice medium were adjusted to different pH values and inoculated. From pH 2.5 to 3.4 no growth was observed, but at pH values of 3.5 and 3.6 growth sometimes occurred. From pH 3.7 to 8.0 growth was vigorous, although the onset of growth was delayed at either end of this range. Of twenty-three naturally sweet sick ciders, twenty-one were within the range of pH 3.7–4.1, while the remaining two ciders were on the tolerance threshold of pH 3.4–3.6.

Table 2. *Fermentation of glucose by the cider-sickness bacillus*

	Glucose (g./100 ml.)		Alcohol produced (g./100 ml.)	Ratio: alcohol glucose, (g./g. %)
	Sterile medium	After fermentation		
Strain 1	4.58	0.004	2.22	48.5
		0.009	2.23	48.8
Strain 2	4.58	0.016	2.25	49.4
		0.016	2.25	49.4

## DISCUSSION

In their sugar reactions and other properties, both strains of the cider-sickness bacillus were almost identical with *Achromobacter anaerobium*, and these organisms differed principally from *Termobacterium mobile* in their inability to ferment sucrose (see Table 1). All three organisms fermented glucose almost quantitatively to ethanol and carbon dioxide (see Table 2, Kluwyer & Hoppenbrouwers, 1931; Shimwell, 1937). When inoculated into pasteurized cider *A. anaerobium* and *T. mobile* grew and fermented the sugars vigorously. The resulting spoiled ciders were sampled in random order by an experienced panel of cider-tasters. They agreed unanimously that the flavours which these organisms induced in cider were similar although not identical with cider sickness. It seems, therefore, that all three organisms are closely related and should be placed in the same genus.

Lindner (1928) named his original isolate *Termobacterium mobile*; Kluwyer & Hoppenbrouwers (1931), who studied this organism in some detail and discussed its taxonomy, considered that it should be called *Pseudomonas lindneri* under the Lehmann-Neumann system. Kluwyer & van Niel (1936), in their paper on a natural system for the classification of bacteria, proposed a new genus *Zymomonas* in the tribe Pseudomonadeae and that Lindner's organism should be the type species (*Zymomonas mobile*). Shimwell (1937) isolated a similar organism from beer and placed it in the genus *Achromobacter*. *Bergey's*



*Manual of Determinative Bacteriology* (1948) adopted *Pseudomonas lindneri* as the name for Lindner's isolate, with *Termobacterium mobile* as a synonym, but this classification made no mention of the proposed new name *Zymomonas mobile*. Later Shimwell (1950), who was not satisfied with the generic names *Achromobacter* or *Pseudomonas*, proposed the creation of a new genus *Saccharomonas* in the tribe Pseudomonadeae to contain his isolate from beer and Lindner's organism. However, since Kluyver & van Niel have precedence for the name *Zymomonas* for these same organisms, it is proposed to adopt their nomenclature.

The chief difference observed between *Zymomonas anaerobia* Shimwell, and the cider-sickness bacillus, was in the change which the two organisms produced in cider. Although this is important from a technical point of view, it is of minor significance in bacteriological classification. *Z. mobile* Lindner differs not only in its effect on cider, but also in its ability to ferment sucrose. It is considered, therefore, that the cider-sickness bacillus is different from *Z. mobile* but that it resembles *Z. anaerobia* so closely that it can be regarded as a variety of that species. Prof. B. T. P. Barker, in a private communication, has suggested the variety name should be *pomaceae*. It is proposed then that the cider-sickness bacillus be called *Z. anaerobia* var. *pomaceae*. A subculture of *Z. anaerobia* var. *pomaceae* strain 1 has been deposited at the National Collection of Industrial Bacteria, Teddington, Middlesex, England.

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## The Use of $\beta$ -Glucosides in Classifying Yeasts

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**SUMMARY:** The  $\beta$ -glucosidase activity of 119 strains of yeast was studied with aesculin, arbutin, salicin or cellobiose as substrates. A medium which was sufficient for good growth was in many cases not sufficient to allow the development of  $\beta$ -glucosidase activity. A yeast capable of splitting one  $\beta$ -glucoside did not necessarily hydrolyse another. It is suggested that yeasts may contain different  $\beta$ -glucosidases. There is evidence that, grown under suitable conditions, most yeasts are capable of hydrolysing aesculin.

The ability to hydrolyse various  $\beta$ -glucosides has been used in classifying yeasts. Four  $\beta$ -glucosides have been used: aesculin, arbutin, salicin and cellobiose; all are  $\beta$ -D-glucopyranosides (see Ingram, 1955). The three phenolic  $\beta$ -glucosides on hydrolysis yield phenols, the presence of which may be shown by a suitable colour reaction such as that with ferric salts. The criterion used to determine the ability to hydrolyse cellobiose has been growth with this sugar as the sole source of carbon, it being assumed that the disaccharide is split before utilization for growth.

Stelling-Dekker (1931) was the first to use a  $\beta$ -glucoside for classifying yeasts, when she tested strains of *Pichia* and *Hansenula* against aesculin by streaking the yeast on yeast-water agar containing 0.5 % aesculin and a drop of iron ammonium citrate solution. Diddens & Lodder (1942) used arbutin plates instead of aesculin for classifying asporogenous yeasts, because arbutin was cheaper and gave the same results (Dr J. Lodder, private communication). Their medium contained 5 g. arbutin, 1 l. yeast water, 1 drop  $\text{FeCl}_3$  solution, 20 g. agar, and was autoclaved; the results were observed after 2, 4 or 6 days. Except for adding a drop of  $\text{FeCl}_3$  solution to each plate, the same method was followed by Lodder & Kreger-van Rij (1952) in classifying all types of yeast; they suggested (1954) that aesculin might be used as an alternative to arbutin. More recently, cellobiose and salicin have been used for identifying yeasts (Wickerham, 1951; Wiles, 1953); in both cases, growth was the criterion for utilization. Salicin was used by Gordon (1905) for differentiating streptococci and Henry & Auld (1905) showed that, like arbutin, it was hydrolysed by baker's yeast. Table 1 gives a chronological summary of some contributions to this subject.

Hitherto, there has been no attempt to compare the ability of a large number of yeasts to split several  $\beta$ -glucosides, although Lodder & Kreger-van Rij (1954) indicated that a yeast which splits arbutin also splits aesculin, and vice versa. Previous workers did not discuss their techniques at all critically.

Table 1. *Chronological summary of some studies concerned with  $\beta$ -glucoside splitting by micro-organisms*

Date	Author	Contribution
1893	Bourquelot	Extract from <i>Aspergillus niger</i> split amygdalin, coniferin and salicin
1903	Kobert	Aesculin, arbutin and salicin split by invertebrate enzymes
1905	Gordon	Differentiation of streptococci, using acid production with 1 % coniferin or salicin
1905	Henry & Auld	Baker's yeast capable of splitting amygdalin, arbutin and salicin
1906	Gonnermann	Certain intestinal bacteria split arbutin: tested with ammoniacal silver nitrate
1907	Beijerinck	Aesculin (0.1 %) + 1 drop ferric citrate in whey agar for studying milk-souring organisms
1909 (a & b)	Harrison & Van der Leek	Aesculin bile salt medium for isolating <i>Escherichia coli</i> and <i>Salmonella typhi</i> . Reaction only in glucose-free medium. Results in second paper suggest phosphate interferes
1917	Bau	<i>Saccharomyces ludwigii</i> and Saaz yeast both active against amygdalin
1917	Gosio	For diagnosing dysentery bacilli: arbutin added 1/200 to ordinary agar medium; hydroquinone thought to blacken by oxidation in air
1920	Van Steenberghe	Aesculin (0.1 %) + ferric citrate (0.1 %) in yeast water agar for classifying milk organisms
1924	Weintraub	Arbutin, salicin and $\beta$ -methyl glucoside for <i>E. coli</i> ; acid and gas production
1929	Weatherall & Dible	Distinguished between splitting aesculin and growing in presence of bile
1929 & 1930	Weidenhagen	Top and bottom yeasts split both $\beta$ -glucoside links of amygdalin and cellobiose
1931	Stelling-Dekker*	Aesculin (0.5 %) + 1 drop ferric citrate in yeast water agar: used for <i>Pichia</i> and <i>Hansenula</i> spp.
1932	Neuberg & Hofmann	Study of yeasts $\beta$ -glucosidase: splitting of amygdalin, arbutin, cellobiose and salicin by extract from <i>Saccharomyces fragilis</i>
1935	Castellani & Douglas	Arbutin (1 %) agar (no added iron) for bacteria: explanation of reaction not known
1937	Castellani*	Arbutin agar (?no added iron) for pathogenic yeasts
1942	Diddens & Lodder*	Arbutin (0.5 %) agar + $\text{FeCl}_3$ solution (1 drop/l.) for anascopogenous yeasts
1948	Wickerham & Burton*	Cellobiose and salicin for yeast growth tests in liquid culture
1952	Lodder & Kreger-van Rij*	Arbutin (0.5 %) agar + $\text{FeCl}_3$ solution (1 drop/plate) for most yeasts
1953	Wiles*	Aesculin, cellobiose, salicin for yeast growth tests in liquid culture
1954	Lodder & Kreger-van Rij*	Arbutin and aesculin tests are alternative

Authors marked with asterisk (\*) were concerned with yeast taxonomy.

It seemed worth while, therefore, to develop methods for studying yeast  $\beta$ -glucosidases under standardized and relatively well-understood conditions. With rapid techniques for detecting  $\beta$ -glucosidase activity, the authors have attempted to answer the following questions:



- (1) Will a yeast which hydrolyses one  $\beta$ -glucoside hydrolyse others?
- (2) Are differences between the rates of hydrolysis of various  $\beta$ -glucosides of value in classifying yeasts?
- (3) Will tests with one  $\beta$ -glucoside alone give maximum information about  $\beta$ -glucosidase activity for purposes of classification, and if so, which test is the most sensitive and convenient, and how quickly can a consistent result be obtained?

## METHODS

### *Media*

- (1) Malt yeast glucose peptone agar (MYGPA) (Wickerham, 1951), pH 6.0.
- (2) Honey yeast glucose potato agar: (HYGSA) (cf. Lochhead & Farrell, 1931; Ingram, 1950; Scarr, 1951): honey (Cambridgeshire) 300 g.; glucose 200 g.; Difco Bacto potato dextrose agar 40 g.; fresh yeast water (Lodder & Kreger-van Rij, 1952), 1 l.; autoclaved at 115° for 15 min.; final pH 5.5.
- (3) Honey yeast glucose peptone agar (HYGPA). As for HYGSA, but 5 g. Difco Bacto peptone and 5 g. Difco Bacto agar in place of potato dextrose agar; final pH 5.5.
- (4) The carbon-free chemically defined medium of Barnett & Ingram (1955), was used (i) without additions (A 2); (ii) with 1 % (w/v) glucose and 2 % (w/v) agar (A 2G agar): vitamins and glucose were Seitz-filtered and added aseptically.
- (5) A 2 + 0.05 % (w/v) aesculin, Seitz-filtered.
- (6) A 2 + 0.5 % (w/v) arbutin, Seitz-filtered.
- (7) A 2 + 0.5 % (w/v) salicin, Seitz-filtered.
- (8) A 2 + 0.1 % (w/v) cellobiose, Seitz-filtered.
- (9) Arbutin agar (Lodder & Kreger-van Rij, 1952).
- (10) Aesculin agar, prepared as described (a) by Stelling-Dekker (1931) and (b) by Van Steenberghe (1920).
- (11) Breakdowns of MYGPA: made up as for MYGPA, but omitting various constituents in turn: 'MYGA', 'MYPA', 'MGPA', 'YGPA', 'YGA' and 'YA' (see Table 6 for pH values).
- (12) Difco Bacto potato dextrose agar: (SDA), pH 5.8.
- (13) Difco Bacto wort agar, with addition of 0.5 % (w/v) Bacto agar (WA) pH 5.2.
- (14) Hartley's meat digest agar (Mackie & McCartney, 1949; NA); pH 7.5.
- (15) Malt extract agar (Blakeslee, 1915; MEA): pH 5.9.
- (16) Malt yeast glucose potato agar (MYGSA). Difco Bacto malt extract broth 15 g.; glucose 10 g.; Difco Bacto potato dextrose agar 40 g.; Difco Bacto agar 5 g.; fresh yeast water 1 l. Autoclaved at 115° for 15 min.; final pH 5.7.

*Glucosides.* Aesculin and arbutin from L. Light and Co. Ltd., salicin and cellobiose from T. Kerfoot and Co. Ltd. The names of these glucosides are abbreviated to AE, ARB, SAL and CEL in the tables.

*Compounds for analysis* were of Analar quality or of the highest quality available.

*Organisms.* Most of the strains of yeast used in these experiments were selected for their interest with respect to food microbiology, therefore not all genera are represented. The names and sources of the strains are given in Table 2. Throughout this paper, the names used are those adopted by Lodder & Kreger-van Rij (1952); except for two strains with names which these authors do not consider and alternative names given in Table 2. Stock cultures were maintained at  $+1^{\circ}$  on slopes of A2G agar, MYGPA or HYGSA. These stocks were subcultured every 6 months (see Kirsop, 1954).

#### *Plate techniques*

*Arbutin.* Every strain was tested with the arbutin plate technique of Lodder & Kreger-van Rij (1952). Fresh yeast extract was prepared as these authors prescribed and 0.5% (w/v) arbutin and 2% (w/v) agar added. The medium was autoclaved at  $120^{\circ}$  for 15 min. The melted medium was poured into Petri dishes each of which contained one drop of sterile 1% (w/v)  $\text{FeCl}_3$  solution, and was thoroughly mixed (Lodder & Kreger-van Rij do not say what strength  $\text{FeCl}_3$  solution they used).

*Aesculin.* The method of Van Steenberghe (1920) was used (see Table 1). A strip of agar was cut out across the centre of the plates with a sterile scalpel, giving two areas between which the aglycone could not diffuse (Kluyver & Custers, 1940). One area was inoculated and the other served as a non-inoculated control.

Attempts to use the medium of Stelling-Dekker (1931) were unsuccessful, perhaps because of uncertainty about the amount of iron to include. Dr G. C. Ingram (personal communication), with bacteria, found the concentration of iron in aesculin plates to be critical.

*Growth experiments.* The growth of certain strains was measured with cellobiose as the only source of carbon (Table 2). Shaken cultures in T-tubes were used (Barnett & Ingram, 1955); the inocula were cultivated in Roux bottles on A2G agar (except for NCYC 245 which had to be grown on MYGPA).

*Preparation and use of the suspensions of organisms.* For each experiment, a fresh slope was inoculated from the stock culture. After incubation for 3 days the organisms were washed off the slope with medium A2 and the entire crop used to inoculate a Roux bottle containing about 100 ml. of A2G agar, MYGPA, HYGSA or HYGPA. After 3 days of incubation at  $25^{\circ}$ , the cultures were harvested in 10 ml. of medium A2 and transferred to 1 oz (25 ml.) screw-capped bottles by means of sterile pipettes.

#### *Estimation of splitting activity*

In the routine experiments 2 ml. of suspension of organism was added aseptically to 2 ml. of medium A2, containing the required glucoside, in a T-tube (Barnett & Ingram, 1955). One T-tube was set up for each glucoside for each yeast strain; in addition, for each group of experiments there were control tubes without added yeast. The tubes were shaken (*c.* 2 cyc./sec. 5 cm.

**Table 2.** *The reactions of 119 strains of yeast to four  $\beta$ -glucosides*

Classification of Lodder & Krieger-van Rij (1952)	Other name given previously	Strain	Quantitative tests with suspension of organisms grown on										Arbutin plate reactions for species given by Lodder & Krieger- van Rij (1952)		
			A2G agar			Complex medium									
			No. of expts.	Aesculin	Arbutin	Salicin	Cello- biase	No. of expts.	Aesculin	Arbutin	Salicin	Cello- biase		Aesculin plates	Arbutin plates
<i>Candida guilliermondii</i>	<i>Torula fermentati</i>	NCYC 145	1	++	+	+	+	+	+	+	2	+	+	+	+
<i>C. krusei</i>		NCYC 338	1	+	-	-	-	-	-	-	1	+	+	-	-
<i>C. mycoderma</i>		NCYC 335	1	-	-	-	-	-	-	-	1	+	+	-	-
<i>C. tropicalis</i>		NCYC 4	2	+(3)	-	+	+	+	+	+	1	+	+	-	-
<i>C. utilis</i>	<i>C. vulgaris</i>	NCYC 359	1	++	+	+	+	+	+	+	0*	.	.	+	+
<i>Cryptococcus laurentii</i>	<i>Torulopsis utilis</i> var. major	NCYC 139	1	++	+	+	+	+	+	+	.	.	.	.	- or W
<i>Debaryomyces hansenii</i>	<i>Torula aurea</i>	LTS 1	2	++	+(3)	+	+	+	+	+	1	+	+	+	V
<i>Hansenula anomala</i>		Ginkerman 416	2	++	+	+	+	+	+	+	1	+	+	+	V
<i>H. saturnus</i>	<i>W. illia anomala</i>	NCYC 18	1	++	+	+	+	+	+	+	.	.	.	.	+
<i>H. saturnus</i>	<i>H. saturnus</i>	NCYC 22	1	++	+	+	+	+	+	+	.	.	.	.	+
<i>H. suavis</i>	<i>Pichia suavis</i>	NCYC 57	1	++	+	+	+	+	+	+	.	.	.	.	+
<i>H. subpelliculosa</i>	<i>Hansenia apiculata</i>	NCYC 16	1	++	+	+	+	+	+	+	.	.	.	.	+
<i>Kloeckera africana</i>	<i>Kloeckera santarosensis</i>	NCYC 26	4	++	+	+(2)	+	+	+	+	+(3)	.	.	+	+
	<i>Pseudosaccharomyces magnus</i>	NCYC 58	.	+	+	+	+	+	+	+	1	+	+	+	-
<i>K. antillarum</i>	<i>Kloeckera willii</i>	NCYC 37	1	++	-	+	+	+	+	+	0	+	+	+	-
<i>K. apiculata</i>		Wiles T70	1	++	++	++	++	++	++	++	.	.	.	.	+
		LTS 5	1	++	++	++	++	++	++	++	.	.	.	.	+
	<i>K. brevis</i>	NCYC 245	1	++	+	+	+	+	+	+	1	+	+	+	-
<i>Pichia fermentans</i>	<i>Pichia kluyveri</i>	NCYC 328	1	++	+(2)	.	+	+	+	+	1	+	+	+	.
<i>P. membranefaciens</i>		NCYC 246	1	-	-	-	-	-	-	-	1	+	+	+	.
	<i>P. membranefaciens</i>	NCYC 54	4	-	-	-	-	-	-	-	5	+	+	+	-
	<i>P. membranefaciens</i>	NCYC 55	1	-	-	-	-	-	-	-	1	+	+	+	.
	<i>Zygosaccharomyces chevalieri</i>	CBS 240	5	-	-	-	-	-	-	-	4	+	+	+	.
<i>P. polymorpha</i>		NCYC 56	3	++	+	+	+(2)	+	+	+	+	+	+	+	+
<i>Rhodotorula glutinis</i>		NCYC 59	1	++	-	+	+	+	+	+	0	+	+	+	?
<i>R. rubra</i>		NCYC 142	1	++	+	+	+	+	+	+	0*	.	.	+	+
<i>R. rubra</i> var. <i>longa</i>		CBS 326	1	++	+	+	+	+	+	+	0*	.	.	+	+
<i>Saccharomyces acidifaciens</i>	<i>Z. acidifaciens</i>	CBS 749	1	+	+	+	+	+	+	+	1	+	+	+	+
		DBDR 427	1	+	-	-	-	-	-	-	.	.	.	.	-
		ATCC 8766	2	++	+	+	+	+	+	+	.	.	.	.	.
<i>Z. mandshuricus</i>		CBS 685	1	+	-	-	-	-	-	-	0†	+	+	+	.
		ATCC 2602	1	+	-	-	-	-	-	-	1	+	+	+	.
<i>Z. bailii</i>		CBS 680	2	+	-	-	-	-	-	-	1	+	+	+	.
<i>Z. bisporus</i>		ATCC 8099	1	-	-	-	-	-	-	-	2	+	+	+	.
		CBS 702	2	-	-	-	-	-	-	-	1	+	+	+	.
<i>S. bailii</i>		NCYC 171	1	-	-	-	-	-	-	-	1	+	+	+	.
<i>S. bisporus</i>		NCYC 74	1	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	1	+	+	+	.
		NCYC 324	1	-	-	-	-	-	-	-	1	+	+	+	.
<i>S. carlsbergensis</i>		NCYC 343	1	-	-	-	-	-	-	-	1	+	+	+	.
		NCYC 355	1	-(3)	-(2)	-(2)	-(2)	-(2)	-(2)	-(2)	1	+	+	+	.

Table 2 (cont.)

Classification of Lodder & Kregen-van Rij (1952)	Other name given previously	Quantitative tests with suspension of organisms grown on										Arbutin plate reactions for species given by Lodder & Kregen- van Rij (1952)	
		A 2G agar					Complex medium						
		No. of expts.	Aesculin	Arbutin	Salicin	Cello- biose	No. of expts.	Aesculin	Arbutin	Salicin	Cello- biose		Aesculin plates
<i>S. cerevisiae</i>		Strain											
		NCYC 77	—	—	—	1	++	+	0	0	—	—	—
		NCYC 231	— (2)	—	—	1	++	+	+	0	—	—	—
		NCYC 353	—	+	—	2	++	+	+	0	—	—	—
		NCYC 369	—	+	—	1	++	+	+	0	—	—	—
		Cukerman 201	—	—	—	2	++	—	+	0	—	—	—
		Mossel	+	—	—	1	+	—	—	0	—	—	—
	<i>Z. priorianus</i>	NCYC 176	+	—	—	2	++	—	+	0	—	—	—
	<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	NCYC 365	+	—	—	1	+	—	+	0	—	—	—
	<i>S. ellipsoideus</i>	NCYC 93	+	—	—	1	++	—	—	0	—	—	—
		NCYC 94	+	—	—	1	+	—	—	0	—	—	—
	<i>Z. fermentati</i>	CBS 818	—	—	—	1	++	—	+	0	—	—	—
	<i>Z. mongolicus</i>	CBS 705	+	—	—	1	++	—	+	0	—	—	—
<i>S. delbrueckii</i> var. <i>mongolicus</i>		NCYC 161	—	—	—	2	—	—	—	0	—	—	—
<i>S. fermentati</i>	<i>Torulasporea fermentati</i>	CBS 748	—	+	—	2	—	—	+	0	—	—	—
<i>S. florentinus</i>	<i>Z. euglycicus</i>	CBS 746	—	—	—	2	— (1)	—	+	0	—	—	—
	<i>Z. florentinus</i>	NCYC 172	— (3)	—	—	2	—	—	+	0	—	—	—
		CBS 397	+	+	+	1	0*	—	—	—	—	—	—
<i>S. fragilis</i>		PHAFF 351	+	+	+	1	0*	—	—	—	—	—	—
		CBS 739	+	+	+	1	— (1)*	—	+	0	—	—	—
<i>S. lactis</i>	<i>Z. casei</i>	CBS 683	+	+	+	2	—	—	+	—	—	—	—
	<i>Z. lactis</i>	CBS 743	+	+	+	1	+	—	+	—	—	—	—
	<i>Z. versicolor</i>	ATCC 2628	+	+	+	1	+	—	+	—	—	—	—
		NCYC 111	+	—	—	1	+	—	—	—	—	—	—
<i>S. marxianus</i>		NCYC 243	+	+	+	1	+	—	—	—	—	—	—
	<i>S. macedoniensis</i>	CBS 745	+	+	+	1	+	—	—	—	—	—	—
	<i>Z. ashbyi</i>	CBS 712	+	+	+	1	0*	—	+	0	—	—	—
	<i>Z. marxianus</i>	CBS 736	+	—	—	1	+	—	+	—	—	—	—
	<i>Z. melis</i>	DBDR D1H	+	+	+	1	+	—	+	—	—	—	—
		CBS 740	+	+	+	1	+	—	+	—	—	—	—
<i>S. melis</i>	<i>Z. nectarophilus</i>	DBDR B2	+	+	+	1	+	—	+	—	—	—	—
		ATCC 8362H	+	+	+	1	+	—	+	—	—	—	—
		DBDR 155Y H2	+	+	+	1	+	—	+	—	—	—	—
		DBDR S3 B2H	+	+	+	1	+	—	+	—	—	—	—
	<i>Z. perspicillatus</i>	ATCC 2629	+	+	+	1	+	—	+	—	—	—	—
	<i>Z. raveniatis</i>	DBDR Z7 H2	+	+	+	1	+	—	+	—	—	—	—
	<i>Z. richteri</i>	CBS 742	+	0	—	1	+	—	+	—	—	—	—
		DBDR M1	+	—	—	1	+	—	+	—	—	—	—
<i>S. pastori</i>	<i>Z. pastori</i>	CBS 704	+	—	—	3	+	—	+	—	—	—	—
		NCYC 175	+	—	—	2	+	—	+	—	—	—	—
	<i>Z. pini</i>	CBS 744	+	+	+	2	+	—	+	—	—	—	—
		Cukerman 401	+	+	—	1	+	—	+	—	—	—	—
<i>S. rosei</i>		CBS 728	—	—	—	1	—	—	—	0	—	—	—
	<i>Z. globiformis</i>		—	—	—	1	—	—	—	0	—	—	—

— or VW



Table 2 (cont.)

Classification of  
Lodder & Kregger-van Rij  
(1952)

Other name  
given previously

Strain

No. of  
expts.

A2G agar

Complex medium

Arbutin plate  
reactions for  
species given  
by Lodder  
& Kregger-  
van Rij  
(1952)

Classification of Lodder & Kregger-van Rij (1952)	Other name given previously	Strain	No. of expts.	A2G agar				Complex medium				Arbutin plates	Aesculin plates	Cello- biase	Arbutin plates
				Aesculin	Arbutin	Salicin	Cello- biase	Aesculin	Arbutin	Salicin	Cello- biase				
<i>S. rouzii</i>	<i>Z. cavaiae</i> var. <i>beauverie</i> <i>Z. gracilis</i> <i>Z. gracilis</i> var. <i>italicus</i> <i>Z. major</i> <i>Z. major</i> var. <i>threntensis</i> <i>Z. major</i> st. <i>salsus</i> <i>Z. nadsomii</i> <i>Z. nussbaumeri</i> <i>Z. rugosus</i>	NCYC 381	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 710	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 731	2	-	-	-	0	+	+	-	0	-	-	-	-
		CBS 732	1	-	-	-	0	+	+	-	0	-	-	-	-
		ATCC 26223	2	-	-	-	0†	+	+	-	0	-	-	-	-
		CBS 687	2	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 689	1	-	-	-	0	+	+	-	0	-	-	-	-
		CBS 688	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 686	1	+	-	-	0	+	+	-	0	-	-	-	-
		NCYC 174	1	-	-	-	0	+	+	-	0	-	-	-	-
<i>S. rouzii</i> var. <i>polymorphus</i>	<i>Z. variabilis</i> <i>Z. vini</i> <i>Z. daitensis</i> <i>Z. anaeoboides</i> <i>Z. barteri</i>	CBS 738H	1	-	-	-	0	+	+	-	0	-	-	-	-
		DBDR J7	1	-	-	-	0	+	+	-	0	-	-	-	-
		CBS 741	1	+	-	-	0	+	+	-	0	-	-	-	-
		ATCC 4898	1	+	-	-	0	+	+	-	0	-	-	-	-
		DBDR Y 139	2	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 730	1	+	-	-	0	+	+	-	0	-	-	-	-
		ATCC 2619	1	+	-	-	0	+	+	-	0	-	-	-	-
		DBDR Z1	1	+	-	-	0	+	+	-	0	-	-	-	-
		ATCC 10687	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 1085	1	+	-	-	0	+	+	-	0	-	-	-	-
<i>S. rouzii</i> var. <i>polymorphus</i>	<i>Z. citrus</i> <i>Z. felineus</i> <i>Z. polymorphus</i>	CBS 706	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 711	1	+	-	-	0	+	+	-	0	-	-	-	-
		DBDR Z3	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 678	1	+	-	-	0	+	+	-	0	-	-	-	-
		NCYC 170	1	+	-	-	0	+	+	-	0	-	-	-	-
		ATCC 2606	1	+	-	-	0	+	+	-	0	-	-	-	-
		DBDR Z4	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 733	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 734	1	+	-	-	0	+	+	-	0	-	-	-	-
		ATCC 2624	1	+	-	-	0	+	+	-	0	-	-	-	-
<i>Schizosaccharomyces octosporus</i>	<i>Torula homii</i> <i>Trichosporon cutaneum</i> <i>None</i> <i>None</i>	CBS 727	1	+	-	-	0	+	+	-	0	-	-	-	-
		CBS 713	1	+	-	-	0	+	+	-	0	-	-	-	-
		SCARR	1	+	-	-	0	+	+	-	0	-	-	-	-
		LTS 3	2	+	+	+	-	+	+	-	+	+	+	+	+
		NCYC 163	1	-	-	-	-	-	-	-	-	-	-	-	-
		NCYC 444	1	-	-	-	-	-	-	-	-	-	-	-	-
		ATCC 11003	1	-	-	-	-	-	-	-	-	-	-	-	-
		ATCC 11388	1	-	-	-	-	-	-	-	-	-	-	-	-

NOTES. Media. The complex medium in each case was MYGPA, except where H or H2 is given by the strain number, indicating HYGSA or HYGPA respectively. Symbols. The symbols +, -, for aesculin, arbutin and salicin results, are explained in Table 10. 0 = no test; V = variable; W = weak; VW = Very weak. No. of experiments. Where the number of tests is not the same for a particular glucoside as that given in the appropriate column, the correct figure is indicated in brackets. Cellobiase growth response: \* positive; † negative.

Strains. ATCC: American Type Culture Collection. CBS: Centraalbureau voor Schimmelcultures (Delft, Holland). Strain numbers given by Mrs N. J. W. Kregger-van Rij. Ciukerman: from Dr I. Ciukerman, Agricultural Research Institute, Rehovot, Israel. Nos. 1 to 3 isolated at LTS, named by CBS. LTS 5 isolated by Lowings (1956). Mossel: from Dr D. A. A. Mossel, Central Institute for Nutrition Research Station, Cambridge (England). Phaff: from Dr H. J. Phaff, University of California. Scarr: from Dr M. P. Scarr, Messrs Tate and Lyle Ltd. (England).

travel) at 25° for 20 hr. The contents of the T-tubes were then centrifuged at *c.* 250 *g* for 5 min., and the supernatant treated as follows:

*Aesculin.* The supernatant fluid from each tube was poured separately into a test tube containing 5 ml. ethyl acetate. The contents of each tube were shaken thoroughly and allowed to stand for *c.* 30 min.; 3 ml. of ethyl acetate layer was added to 5 ml. of 1.5 M-Na<sub>2</sub>CO<sub>3</sub> in a separating funnel and shaken. The colour of the lower (aqueous) layer was measured in  $\frac{5}{8}$  in. diam. tubes at 450 m $\mu$ . (using the control as a blank) by means of a diffraction-grating spectrophotometer (Unicam SP 350; the instrument did not give a satisfactory response at wavelengths much below 450 m $\mu$ .), and at 400 and 450 m $\mu$ . in a Hilger 'Uvispec' spectrophotometer. This technique was described by Barnett & Swain (1956). The 4 ml. solution in which the yeast was suspended contained 1 mg. aesculin, capable of liberating approximately 500  $\mu$ g. aesculetin. Table 3 shows that, for purposes of classification, the results may be assessed in three arbitrary groups. A 5 % degree of splitting (25  $\mu$ g. aesculetin) is about the lowest degree which gives comparable replications. Though it is practicable to detect as little as 5  $\mu$ g. aesculetin, errors of measurement at this concentration are high. As can be seen from Fig. 1*a*, Beer's law is obeyed approximately up to *c.* 25 % splitting. Higher concentrations of aesculetin could have been determined after dilution but this was not done in the routine determinations.

Table 3. *Quantitative assessment of  $\beta$ -glucoside splitting*

Glucoside	Wave-length (m $\mu$ .)	Assessment of degree of splitting	Amount of splitting (%)	Total amount of aglycone liberated ( $\mu$ g.)	Colorimeter readings
Aesculin	450	—	< 5	< 25	< 0.1
		+	5–25	25–125	0.1–0.3
		++	> 25	> 125	> 0.3
Arbutin	470	—	< 0.5	< 20	< 0.24
		+	0.5–25	20–1000	0.24–0.8
		++	> 25	> 1000	> 0.8
Salicin	640	—	< 0.5	< 20	< 0.05
		+	0.5–25	20–1000	0.05–0.67
		++	> 25	> 1000	> 0.67

Measurements for arbutin above 1 % are approximate.

*Arbutin (a)* To 2 ml. of the supernatant fluid (or control) were added 2 ml. 0.5 % (w/v) phloroglucinol solution and 2 ml. N-NaOH. After 30 min. the solution was well shaken and the colour measured at 470 m $\mu$ . in  $\frac{5}{8}$  in. diam. tubes with the diffraction spectrophotometer, using the control as a blank (Porteous & Williams, 1949). The aglycone (hydroquinone) is probably oxidized by oxygen in the solution to *p*-quinone, which reacts with the keto-form of phloroglucinol to give the coloured compound. This was shown to be likely, because no colour change occurred when the reaction was carried out under nitrogen in a Thunberg tube. At concentrations of hydroquinone > 10  $\mu$ g./ml. the method was unreliable (Fig. 1*b*). This was probably because there was a deficiency of oxygen at higher concentrations of hydroquinone, and the

excess hydroquinone reacted with the coloured compound. Although it would have been possible to measure larger quantities of hydroquinone by dilution, this was not done as a matter of routine. With this technique it is practicable to detect in each tube quantities of hydroquinone  $< 4 \mu\text{g.}$  (c. 0.1 % split).

(b) About 0.5 ml. of the original supernatant fluid was added to an equal volume of modified Tollen's reagent (0.5 %, w/v,  $\text{AgNO}_3$  in 0.1 N-NaOH). The colour change was observed visually.

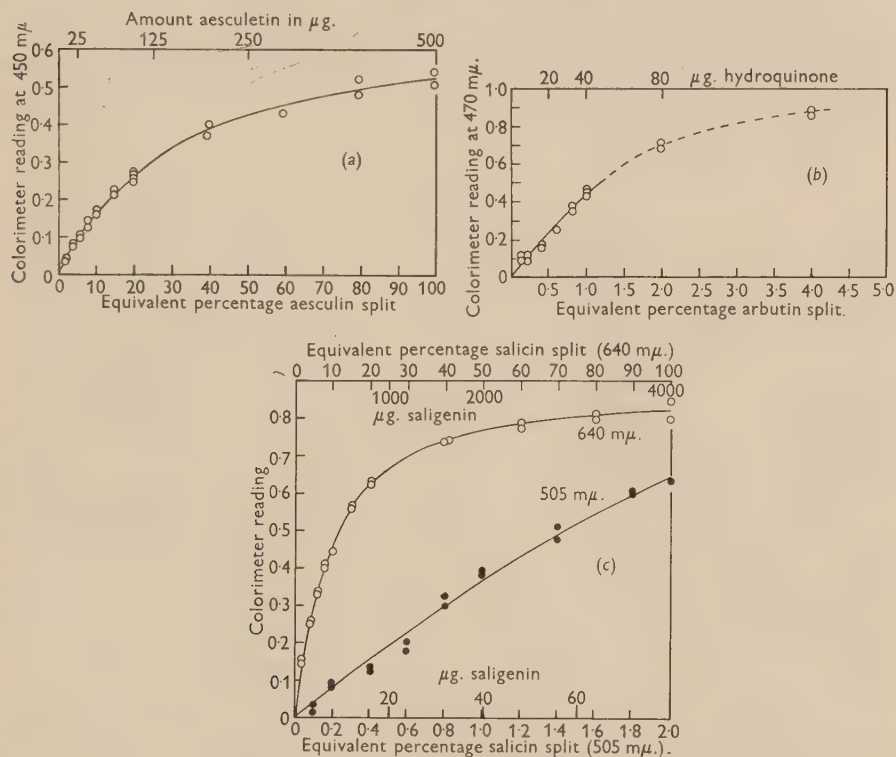


Fig. 1. Calibration curves for (a) aesculin splitting; (b) arbutin splitting; (c) salicin splitting.

**Salicin.** To 2 ml. of the supernatant fluid was added 2 ml. 0.04 % (w/v) 4-amino-antipyrin in 0.1 N-ammonia solution, followed after shaking by 2 ml. 0.2 % (w/v)  $\text{K}_3\text{Fe}(\text{CN})_6$ . The colour was measured spectrophotometrically at 515 and 640 mμ. against the control in  $\frac{5}{8}$  in. diam. tubes. The use of 4-amino-antipyrin for estimating saligenin (see Martin, 1949) is described by Baruah & Swain (to be published).

Using the colorimeter at 515 mμ., readings above about 0.65 were insensitive (Fig. 1c); and, therefore, measurements were carried out also at 640 mμ. In this case, 0.5 % of splitting was taken as the lower limit (see Table 3). Initially 10 mg. salicin were present, capable of liberating about 4 mg. saligenin.

As for aesculetin and hydroquinone, it is practicable to detect as little as 1 μg./ml. saligenin, though 5 μg./ml. is the lower limit used. The concentration

of reagents was chosen to detect readily these lower limits. Thus when more than 25 % salicin was split, dilution was necessary for quantitative analysis; since 25 % is above the limit for a positive result, this was not done.

**Cellobiose.** In preliminary experiments the control tubes, containing yeast alone, as blanks, showed little variation, and were not used subsequently. The supernatant fluids from both tubes were filtered through Whatman no. 50 paper. To 0.5 ml. of filtrate in a  $\frac{5}{8}$  in. test tube, 0.5 ml. 1 % (w/v) 2,3,5-triphenyl-tetrazolium chloride and 0.5 ml. 2N-NaOH were added. The tubes were placed in boiling water for  $30 \pm 2$  sec., removed, 5 ml. 10 % (v/v) glacial acetic acid in *iso*-propanol quickly added, and the mixture cooled to about 20°. The difference in colour between the control and the tube containing sugar was

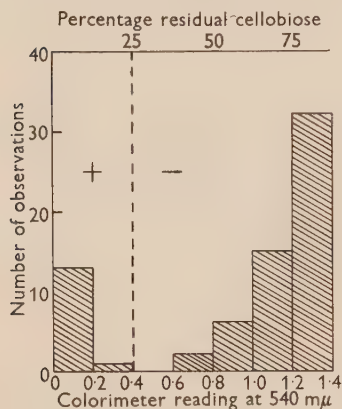


Fig. 2

Fig. 2. Distribution of 69 cellobiose splitting measurements, for strains grown on A2G agar only

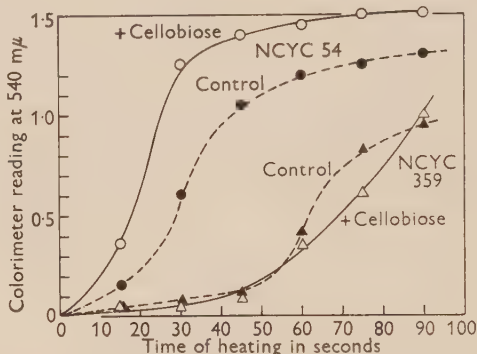


Fig. 3

Fig. 3. Development of formazan with time of heating:

	+ Cellobiose	Control
<i>P. membranaefaciens</i> (NCYC 54) (cellobiose-negative)	○	●
<i>C. utilis</i> (NCYC 359) (cellobiose-positive)	△	▲

measured at 540 mμ. and at 640 mμ. in  $\frac{3}{4}$  in. matched tubes. Since the values for the controls were fairly constant when the yeast was grown on A2G agar, subsequent experiments with this medium were carried out without the use of controls.

The estimation of sugars with tetrazolium was described by Fairbridge, Willis & Booth (1951). The value of  $\lambda$  max.  $\div 485$  mμ. for formazan agrees with their data, but the best response with blanks as above was obtained by reading at 540 mμ. When the readings were greater than 0.65 at this wavelength the depth of colour was also measured at 640 mμ. (see saligenin determination).

There appeared to be introduced into the harvesting medium with the yeast cells substances which reduced tetrazolium, although at slower rates than did cellobiose (see Fig. 3). Consequently, it was necessary to compare formazan formation in media which had contained yeasts + cellobiose with that in media which had contained only yeast. Heating for  $30 \pm 2$  sec. in boiling water gave



the maximum differences between the experimental and control values (Fig. 3). When the yeasts were grown on MYGPA (more especially HYGSA) there was much more of the extraneous reducing substances. Although this method was satisfactory with MYGPA as growth medium, it was not so for yeasts grown on HYGSA. The amount of cellobiose introduced (0.5 mg./ml.) was so chosen that if it was not utilized, it could be detected readily by the above procedure. It was residual cellobiose, which was measured. Considering the relative quantities of yeast and cellobiose involved in these experiments, it has been assumed, *a priori*, that when the yeast splits cellobiose, the resulting glucose is all used. Since the experimental error in this determination is higher than that for the other  $\beta$ -glucosides, no attempt was made to study low activities. Instead, an arbitrary value of 75 % utilization was chosen as the dividing line between +ve and -ve organisms. The distribution of observations given in Fig. 2 shows that most of the yeasts fell either above or below this level.

*Estimation of aglycones.* Solutions of the aglycones aesculetin, hydroquinone and saligenin were made up in medium A 2 at concentrations equivalent to those of the corresponding  $\beta$ -glucosides. Mixtures of each of the glucosides with its corresponding aglycone were made in different proportions to give a series of solutions corresponding with known percentages of hydrolysis of the glucoside (glucose did not interfere with any of the measurements). Aglycone estimations were carried out with solutions containing medium A 2 + glucoside as a blank; and the spectrophotometer readings plotted against the percentage split (Fig. 1). Fig. 1 and Tables 6 and 8 give an idea of the size of errors involved.

*Measurement of concentration of organisms in suspensions.* After estimating the aglycones the yeast from each test tube was resuspended in 50 ml. water and the optical density measured in a  $\frac{3}{4}$  in. diam. colorimeter tube at 580 m $\mu$ . When there were relatively few organisms present, only 10 ml. water was added. For 6 strains these colorimeter readings were compared with haemocytometer counts and dry-weight determinations; this comparison is shown in Table 4. The wavelength chosen was found by trial to be that at which a straight-line relationship was obtained between colorimeter readings and optical density, using serial dilutions over a tenfold range of cell concentration as obtained in these experiments (Table 4).

*Determination of iron in yeast-water* was carried out by the method described by Johnson (1955) with 1 : 10-phenanthroline.

*Extraction of  $\beta$ -glucosidase from baker's yeast.* Baker's yeast (100 g. wet weight, 'Ark' brand) was treated with three successive portions of ice-cold acetone (500 ml.); the resulting powder was dried at room temperature. The acetone powder was extracted with medium A 2 (5 ml./g.) for 20 min. at room temperature and the residue removed by centrifuging. For the determination of activity 2 ml. of the supernatant solution was incubated overnight with the glucoside solution as used for the yeasts, and the liberated aglycone determined as described previously. The extract showed no loss of activity against aesculin when dialysed overnight at +1° against distilled water. When a sample was heated to 100° for 5 min. no measurable activity remained.

Table 4. *Approximate quantities of yeast used in experiments*

Suspensions were prepared as described in the methods section.

	Organisms grown on					
	A2G agar			MYGPA medium		
	Organisms (no./ml.)	Dry wt. organism (mg.)	Colorimeter reading at 580 m $\mu$ .	Organisms (no./ml.)	Dry wt. organism (mg.)	Colorimeter reading at 580 m $\mu$ .
Yeast strains						
<i>Candida utilis</i>						
<i>Rhodotorula rubra</i> var. <i>longa</i>	NCYC 359	.	.	$2.39 \times 10^9$	103	0.66
<i>Pichia membranaefaciens</i>	CBS 326	$1.67 \times 10^9$	67	$3.37 \times 10^9$	116	0.80
<i>Saccharomyces acidifaciens</i>	NCYC 54	$0.39 \times 10^9$	23	$1.63 \times 10^9$	67	0.65
<i>Saccharomyces cerevisiae</i>	CBS 749	$1.43 \times 10^9$	56	$1.18 \times 10^9$	84	0.30
<i>Saccharomyces rouzii</i>	NCYC 77	$0.65 \times 10^9$	25	$1.35 \times 10^9$	59	0.46
	CBS 710	$1.37 \times 10^9$	35	$2.75 \times 10^9$	101	0.37

## RESULTS

The activity shown by each of the 119 strains studied in splitting aesculin, arbutin, salicin and cellobiose is summarized in Table 2. Besides the quantitative estimates, this includes results obtained by using the arbutin plate technique of Lodder & Kreger-van Rij (1952) and some with the aesculin plate method of Van Steenberghe (1920).

*The plate tests*

The arbutin plate test described by Lodder & Kreger-van Rij (1952) gave repeatable results which, as can be seen (Table 2), were usually in agreement with those obtained by these authors; some of the exceptions are discussed later in the light of the quantitative tests. In contrast, aesculin plates prepared by Stelling-Dekker's (1931) method gave variable results, which may have been related to the amount of iron included; this was critical, as has been pointed out. Van Steenberghe (1920) included 0.1 % ferric citrate in his medium, and this technique gave repeatable results (Table 2) though different from those obtained with arbutin. Castellani & Douglas (1935) and Castellani (1937) used an arbutin plate technique without added iron; and the present experiments confirm that the addition of iron is not essential. By contrast with aesculin, it seems that for arbutin plates, the concentration of iron is not critical. Indeed, whereas Diddens & Lodder (1942) advocated one drop of  $\text{FeCl}_3$  solution/litre, Lodder & Kreger-van Rij (1952) recommend one drop/plate (concentration of  $\text{FeCl}_3$  not given in either case). The concentration of iron in the yeast water used was 4.9 mg./l. as determined by the *o*-phenanthroline method. This concentration seems about comparable with that used by Diddens & Lodder (1942), calculated as 2 mg./l. on the assumption that their ferric chloride solution was 5 % (w/v) and that one drop was *c.* 0.1 ml.

*Glucoside concentrations.* The concentration of 0.25 % (w/v) used for salicin and arbutin was chosen since it was of the order of that used in usual  $\beta$ -glucoside tests (e.g. Lodder & Kreger-van Rij, 1952; 0.5 % for arbutin). The concentration of aesculin (0.025 %) was limited by its solubility (given as *c.* 0.15 % in water at 10°; Heilbron & Bunbury, 1946), though Stelling-Dekker (1931) recommended 0.5 %. It is likely that it would have been satisfactory to use each of these glucosides at, say, 0.01 %. As discussed previously, the concentration of cellobiose was minimal.

*Effects of differences in growth media.* It seemed desirable to use a defined medium in order to minimize variation in the experiments (Barnett & Ingram, 1955). Some strains (such as *Kloeckera apiculata* NCYC 245), did not grow well on A2G agar and had to be grown on MYGPA; it was noticed that they all hydrolysed aesculin and tended to be more active than comparable strains grown on A2G agar. A similar difference in activity was also found when strains able to grow on A2G agar were cultivated on MYGPA (Table 5). Of strains grown on A2G agar about 65 % were aesculin-negative; but when they were grown on MYGPA only one of 71 was in this category. Table 5 shows that whenever there was a difference between strains grown alternately on A2G

agar and MYGPA, organisms from the complex medium were the more active. This striking relation between aesculinase and salicinase activity, and the kind of medium on which the organisms had grown, led to an investigation of the  $\beta$ -glucoside splitting capabilities of a yeast grown on 14 different media; the CBS 240 strain of *Pichia membranaefaciens* was used. This is a good example of an organism negative to aesculin when grown on A2G agar, but positive when

Table 5. Summary of differences between patterns of response to  $\beta$ -glucosides for yeasts grown alternately on A2G agar and on MYGPA media

			A2G agar						Σ	
Each figure indicates number of strains			AE	ARB	SAL	+	+	+		
			AE	ARB	SAL	+	+	+		
MYGPA	+	+	+	3	0	1	3	4	3	14
	+	+	-	0	0	0	1	0	1	2
	+	-	+	0	0	2	8	1	14	25
	+	-	-	0	0	0	4	0	11	15
	-	+	-	0	0	0	0	0	0	0
	-	-	-	0	0	0	0	0	0	0
				Σ 3	0	3	16	5	29	56

In this Δ greater splitting follows change from A2G agar to MYGPA medium

AE=aesculin;  
ARB=arbutin;  
SAL=salicin.



Table 6. *β-glucoside splitting activity of Pichia membranaefaciens* (CBS 240) *grown on 14 different media*

Media no. 9 to 14 are breakdowns of MYGPA.

Medium for growth of organism	pH	First experiment										Second experiment														
		Assessment of results					Colorimeter reading*					Concentration of organisms†					Colorimeter reading*					Concentration of organisms †				
							SAL										SAL									
		AE	ARB	SAL	AE	ARB	505 mμ.	640 mμ.	AE	ARB	505 mμ.	640 mμ.	AE	ARB	505 mμ.	640 mμ.	AE	ARB	505 mμ.	640 mμ.						
1 A2G agar	5.0	—	—	—	0	0	0.01	0.03	0.42	0.37	0	0	0.02	0.01	0.42	0.42	0.42	0.44								
2 HYGSA	5.6	+	?	—	0.15	?	0.07	0.04	0.58	0.63	0.09	?	0.06	0.02	0.52	0.46	0.46	0.46								
3 SDA	5.8	+	—	—	0.14	0	0.02	0.01	0.32	0.26	0.11	0.01	0.01	0	0.21	0.20	0.21	0.21								
4 WA	5.2	+	+	—	0.14	0.09	0.06	0.03	0.19	0.22	0.14	0.14	0.02	0.01	0.18	0.21	0.19	0.19								
5 MYGSA	5.7	+	+	—	0.46	0.11	0.11	0.04	0.70	0.66	0.86	0.14	0.07	0.01	0.66	0.66	0.64	0.64								
6 NA	7.5	—	—	+	0	0.03	0.20	0.05	0.06	0.06	0	0.07	0.20	0.02	0.06	0.06	0.06	0.06								
7 MEA	5.9	+	—	—	0.08	0.03	0.02	0.03	0.56	0.47	0.11	0.06	0.02	0.01	0.53	0.53	0.52	0.52								
8 MYGPA	6.0	+	+	—	0.45	0.01	0.06	0.03	0.52	0.52	0.37	0.02	0.05	0.02	0.45	0.50	0.49	0.49								
9 YA	6.7	—	—	—	0.02	0	0.04	0.03	0.06	0.06	0.03	0.01	0.04	0.02	0.04	0.04	0.04	0.04								
10 YGA	6.7	+	+	—	0.45	0.05	0.03	0.03	0.34	0.31	—	—	—	—	—	—	—	—								
11 YGPA	6.8	+	+	—	0.46	0.05	0.06	0.02	0.48	0.48	—	—	—	—	—	—	—	—								
12 MGPA	5.8	+	+	—	0.46	0	0.03	0.03	0.24	0.26	—	—	—	—	—	—	—	—								
13 MYPA	6.3	—	—	—	0.05	0.01	0.05	0.03	0.04	0.04	—	—	—	—	—	—	—	—								
14 MYGA	6.0	+	+	—	0.46	0.06	0.07	0.03	0.50	0.50	—	—	—	—	—	—	—	—								

\* The relations between these colorimeter readings and amount of splitting are indicated in Fig. 1.

† The concentration of organisms here is measured in terms of colorimeter readings comparable with those in Table 4.

AE = aesculin; ARB = arbutin; SAL = salicin.

the presence of a factor in yeast extract. This ' $\beta$ -glucosidase factor' was not a growth factor and has not yet been characterized.

*The effect of washing.* In the routine experiments, as already described, the yeasts were washed from the agar by 10 ml. of medium A 2. This suspension was then used in 2 ml. volumes, each of which was added to 2 ml. of medium A 2 containing, for example, 0.5 % (w/v) aesculin. Table 7 indicates that there was little difference in the response of the organism to aesculin, even when the suspension was washed 3 times. The fluid from the first wash (the initial 10 ml. medium A 2 with which the cells had been harvested) was used in the same way as the suspension, diluting it 1/1 with 0.05 % (w/v) aesculin in medium A 2. The results (Table 7) show that there was some aesculinase activity in this fluid, particularly when the yeast was grown on MYGPA. This might have been due to autolysis which liberated intracellular enzyme, since colonies grown on MYGPA developed faster than those cultivated on A2G agar. MYGP broth alone did not hydrolyse aesculin.

Table 7. *Effect of washing on aesculin splitting by five yeasts grown on two different media*

Each figure is for a different suspension of organisms differently treated as indicated, and represents the quantity of aesculetin liberated (in  $\mu\text{g.}$ ), calculated from colorimeter readings at 450  $m\mu$ . as in Fig. 1.

Organism		Organism grown on					
		A 2G agar			MYGPA		
		Un-washed cells	Washed cells	Liquor from first wash	Un-washed cells	Washed cells	Liquor from first wash
<i>Pichia membranaefaciens</i>	CBS 240	13	13	13	250	238	58
	NCYC 54	13	10	13	225	275	70
<i>Saccharomyces cerevisiae</i>	NCYC 176	90	25	10	225	250	163
<i>S. marxianus</i>	CBS 712	200	250	20	250	250	90
<i>S. pastori</i>	CBS 704	13	38	10	185	238	25

#### *Amount of hydrolysis*

With every strain studied it has been possible to detect some hydrolysis of aesculin: those which were negative when grown on A2G agar were all positive when grown on MYGPA. Only 2 strains (*Saccharomyces mellis* DBDR Z7 and *Schizosaccharomyces octosporus*) are given in Table 2 as aesculin-negative when grown on complex media. The colorimeter readings for these observations were 0.08 and 0.04, respectively, corresponding with the liberation of about 20 and 10  $\mu\text{g.}$  of aesculetin (Fig. 1a). At this level, errors can arise which are of the same order as the values observed; the colour of an alkaline solution containing about 3  $\mu\text{g./ml.}$  aesculetin could be distinguished from that of the control by eye.

In these experiments strains were found which, on both complex and defined media, split almost any proportion of the aesculin present (0.25 mg./ml.), from 0 to nearly 100 %; and there is a tendency for the final degree of splitting to be characteristic for the strain. The same appears true for salicin and arbutin.

In the hope of discovering why some yeasts give a lower degree of splitting than others, replicate experiments were run for 5 strains with different characteristics, aesculetin estimations being made at intervals up to 72 hr. The results (Fig. 4) show that in most cases after 20 hr. of shaking (the time at which analyses were made in the routine experiments), most of the hydrolysis had occurred. Ten A2G agar-grown strains, aesculin-negative at 20 hr., were shaken with aesculin for 120 hr. and of these two passed the positive level of hydrolysis, liberating more than 25  $\mu$ g. aesculetin (*Pichia fermentans* NCYC 246, 120  $\mu$ g.; *Saccharomyces cerevisiae* NCYC 353, 50  $\mu$ g.); the 8 other strains showed no significant change.

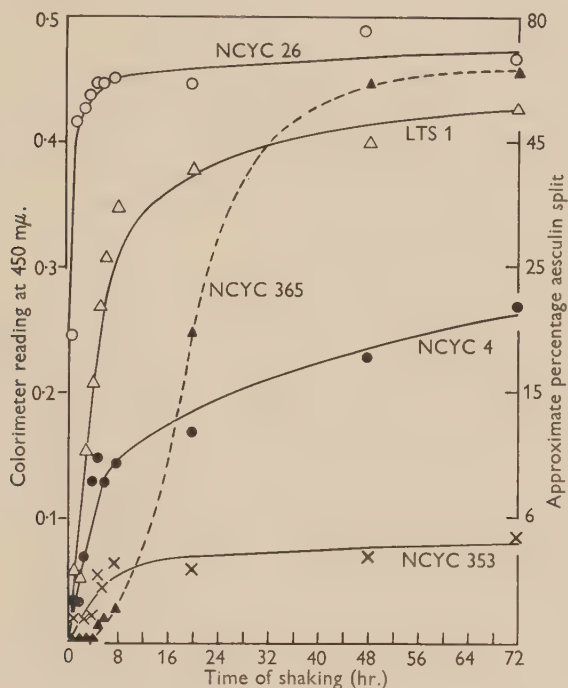


Fig. 4. The course of aesculin splitting by 5 strains over 3 days. Organisms grown on A2G agar. *C. tropicalis*, NCYC 4, ●; *D. kloedenii*, LTS 1, △; *K. africana*, NCYC 26, ○; *S. cerevisiae*, NCYC 353, ×; *S. cerevisiae* var. *ellipsoideus*, NCYC 365, ▲.

It is possible that the differences in the maxima of the curves in Fig. 4 are due to inhibitory activity by aesculetin. If this be so, then aesculetin seriously inhibited hydrolysis at about 3  $\mu$ g./ml. (cf. Fig. 1a) for NCYC 353 and 12  $\mu$ g./ml. for NCYC 4. If the aesculetin simply inactivated the enzyme, these levels may be directly related to the quantity of enzyme which has been synthesized in the organisms before they were introduced into the aesculin medium.

Although a low rate of enzyme synthesis seemed a possible cause of the low activity on a minimal medium, other explanations are possible. For instance, it is conceivable that a specific inhibitor was synthesized when the yeast was

cultivated in these conditions, but not on a richer medium, as for pyrophosphatase in *Proteus vulgaris* (Swartz, Kaplan & Frech, 1956).

The concentration of organisms used in the routine experiments was not strictly controlled, though it was measured turbidimetrically at the end of each experiment (see Tables 4 and 8). The number of organisms involved in each experimental tube was, for those grown on A2G agar, *c.* 2 to  $6 \times 10^9$ ; and for MYGPA experiments, *c.* 4 to  $14 \times 10^9$  (Table 4). The measurements given for replicated experiments in Table 8 indicate that variations in numbers were not usually of importance in determining the results. It is possible to try to put some of the data into terms of molecules split/organism, as advocated by Pirie (1955). Thus, for the data in Table 8, roughly  $6 \times 10^9$  organisms of CBS 240 strain of *Pichia membranaefaciens*, grown on MYGPA, split *c.* 1 mg. aesculin in 20 hr.; i.e. about  $350 \times 10^{11}$  molecules/organism.

The activities shown in Fig. 4 are expressed in Table 9 in terms of molecules/org./sec. during the phase of greatest activity: the errors (chiefly from the estimate of numbers of cells) should be less than a factor of 5. Probably those strains classed as negative split less than 1000 aesculin molecules/org./sec.; those given as *+c.* 1000 to 10,000; and as *++* > 10,000.

#### Assessment of the measurements

The symbols *++*, *+* and *-*, used in Table 2 for indicating results of the quantitative tests for the hydrolysis of the phenolic  $\beta$ -glucosides, are explained in Table 3. It is frequent in microbiology to use *+* and *-* only, but this gives a minimum of information about a quantitative process. With the system used in Table 2, however, it is possible to be fairly certain of the reality of a distinction between *++* and *-*. In the opinion of the present writers, three such categories are the least to be expected from biochemical tests used in classification. After 20 hr. have elapsed, variations in the time at which the experiment is stopped and the analysis carried out probably have little effect on the assessment (Fig. 4). It can be seen that the activity of a strongly aesculin-positive strain (e.g. NCYC 26, *Kloeckera africana*) may be detected after shaking for 1 hr., but a yeast such as NCYC 365 (*Saccharomyces cerevisiae* var. *ellipsoideus*), which gives a curve suggestive of adaptation, would be assessed as negative (*-*) after 8 hr. *+* at 20 hr., and *++* after 2 days. Table 3 shows that the values chosen for the difference between negative and positive are comparable for each glucoside in terms of the amount of aglycone liberated. On the other hand, the dividing line between *+* and *++* was drawn at 25 % splitting in each case.

#### Cellobiose

Results of measuring growth responses to cellobiose as the sole source of carbon are given in Table 2; and they are identical with those of other workers in the four cases where a growth technique has been used (Table 10). The results obtained by the tetrazolium method agree reasonably well, although with the CBS 683 strain of *Saccharomyces lactis* the tetrazolium measurement is probably aberrant. Auxanographic growth responses to cellobiose for three



Table 8. Results of some replicated experiments

Organism	Medium for growth of organism	Aesculin			Arbutin			Salicin		
		Splitting			Splitting			Splitting		
		Assessment	Colorimeter reading*	Concentration of organisms†	Assessment	Colorimeter reading*	Concentration of organisms†	Assessment	Colorimeter reading*	Concentration of organisms†
			450 m $\mu$ .			470 m $\mu$ .			515 m $\mu$ .	
									640 m $\mu$ .	
<i>Pichia membranaefaciens</i>	CBS 240 A2G agar	—	0	0.45	—	0	0.42	—	?	0.46
		—	0	0.42	—	0	0.42	—	0.02	0.44
		—	0	0.42	—	0	0.37	—	0.01	.
		—	0.03	0.45	—	0	0.44	—	0.03	.
		—	0	0.58	—	0	0.45	—	0.03	0.58
<i>Saccharomyces bisporus</i>	MYGPA	++	0.43	0.49	—	0.05?	0.42	—	0.05	0.46
		++	0.39	0.46	—	0.03	0.43	—	?	0.43
		++	0.37	0.45	—	0.02	0.50	—	0.05	0.49
		++	0.45	0.52	—	0.01	0.52	—	0.06	.
		—	0.09	0.29	—	0	0.27	—	0.01	0.27
<i>S. cerevisiae</i>	CBS 702 A2G agar	—	0.07	0.20	—	0	0.22	—	0.01	0.22
		++	0.39	0.52	—	0.03	0.56	—	0.14	0.44
		++	0.37	0.62	—	0	0.50	—	0.19	.
		—	0.07	0.46	—	0	0.46	—	0.01	0.45
		+	0.22	0.20	—	0	0.19	—	0.02	0.22
<i>S. pastori</i>	NCYC 176 A2G agar	++	0.38	0.64	—	0.14	0.66	+	0.51	0.66
		++	0.39	0.58	—	0.20	0.54	+	0.85	.
		—	0.06	0.29	—	0.02	0.30	—	?	0.29
		—	0	0.45	—	0.02	0.40	—	0.03	0.42
		+	0.22	0.66	—	0.05	0.58	—	0.05	0.60
	MYGPA	+	0.13	0.60	—	0.02	0.59	—	0.07	.

\* Relations between colorimeter readings and amount of splitting are indicated in Fig. 1.

† The concentration of organisms here is measured in terms of colorimeter readings comparable with those in Table 4.

Table 9. Rates of aesculin-splitting by five yeasts

See Fig. 4. The organisms were grown on A2G agar.

Organism	Extent of phase of greatest activity (hr.)	Total number of org.* $\times 10^9$	Amount aesculin split (mg.)	$\mu$ g. aesculin split/org./hr. $\times 10^{-9}$	Molecules aesculin split/cell/sec. in active phase $\times 10^3$	Assessment (after 20 hr.)
<i>Candida tropicalis</i>	0-4	6	0-075	3	1-5	+
<i>Kloeckera africana</i>	0-4	2	0-55	70	33	+
<i>Saccharomyces cerevisiae</i>	0-4	2-6	0-02	1-9	0-9	-
<i>S. cerevisiae</i> var. <i>ellipsoideus</i>	8-48	4	0-55	3-4	1-8	+
<i>Debaryomyces hansenii</i>	0-8	2	0-325	20	13-8	++

\* These quantities were contained in the usual 4 ml. of medium in a T-tube.

Table 10. Comparison of results with those of other workers

Organism	Quantitative results (from Table 2)						Results of other workers					
	AE			SAL			Results			References		
	ARB	CEL	CEL	ARB	SAL	CEL	ARB	SAL	CEL	ARB	SAL	CEL
<i>Candida guilliermondii</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>C. krusei</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>C. mycoderma</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>C. tropicalis</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>Hansenula anomala</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>H. saturnus</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>H. subpelliculosa</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>Kloeckera apiculata</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>Pichia fermentans</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>P. membranaefaciens</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>P. polymorpha</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>Saccharomyces carlsbergensis</i>	+	+	+	+	+	+	+	+	+	6	6	6
<i>S. cerevisiae</i>	+	+	+	+	+	+	+	+	+	6	6	6

±, some strains +, others -; +, some strains -, others weak. M: organisms grown on MYGPA; A: organisms grown on A2G agar.

1, Bedford (1942); 2, Lodder &amp; Kreger-van Rij (1952); 3, Stelling-Dekker (1931); 4, Teunissen (1954); 5, Wickerham (1951); 6, Wiles (1953).

species were given by Barnett & Ingram (1955) as: *Saccharomyces bailii* (CBS 680) -ve; *S. marxianus* (CBS 712) +ve; a strain of *S. rouxii* -ve. These results agree with the observations described in the present paper.

#### DISCUSSION

Although the arbutin plate test gives fairly consistent results and may therefore have some use as a crude instrument of sorting, it does not seem to give much useful information about the arbutin-splitting capacities of a yeast. For one thing, it is probably rather insensitive. Some species of *Kloeckera*, for instance, are shown as arbutin-negative, though they can be demonstrated to be quite active splitters of arbutin (Table 2). Further, Lodder & Kreger-van Rij (1952) described all *Kloeckera* species as arbutin-negative, and the National Collection of Yeast Cultures place their strains NCYC 37 of *K. antillarum* and NCYC 328 of *K. apiculata* in the same category. Careful observation of plates with these two strains showed that the brown iron-hydroquinone complex was formed more slowly than its rate of diffusion across the plate and thus no distinct brown area was formed round the colony. When a diagonal strip of agar was cut from the plate with a sterile scalpel and the surface then inoculated on one side only (see Kluyver & Custers, 1940), it soon became obvious that the inoculated side was appreciably darker than the control; this was confirmed by Dr E. O. Morris (personal communication).

In one case studied, the arbutin plate test may have been actually misleading in the opposite way, showing a strong positive result for a 'negative' organism. From Table 2 it can be seen that *Pichia polymorpha* (NCYC 56) was arbutin-negative by the phloroglucinol test when grown on A2G agar or MYGPA. Dr Morris (personal communication) described three arbutin plates for this strain as having a deep brown zone around each colony which was of a black metallic colour. Analysis by the phloroglucinol method of a portion of the agar in the dark zone revealed that no more hydroquinone was liberated than in the case of an 'arbutin-negative' strain such as *Saccharomyces cerevisiae* (NCYC 231). Presumably, therefore, either this strain of *P. polymorpha* was capable of making a coloured complex directly with arbutin; or it split arbutin and made the hydroquinone unavailable by coupling it (or the iron complex) with, for example, an amino acid (cf. pulcherrimin: Walt, 1952).

Further, as for instance Table 5 shows, the nutrition of the growing yeast is critical in determining the result of a  $\beta$ -glucoside test. In the arbutin-plate test, the organisms were grown on yeast water agar, which, as has been seen at least with aesculin, does not appear to be a good medium for developing  $\beta$ -glucosidase activity (Table 6). This point is well illustrated by the activity of the strains of *Saccharomyces cerevisiae*, which were arbutin-plate negative (Lodder & Kreger-van Rij, 1952), but quite active as  $\beta$ -glucoside splitters when grown on MYGPA. Henry & Auld (1905) described baker's yeast (*S. cerevisiae*) as capable of splitting arbutin and salicin (see Table 1). Commercial samples of baker's yeast ('Ark Brand'), substantially free from

bacterial contaminants, were found in the present experiments to be active aesculin-splitters when tested directly without further cultivation; Brunet & Kent (1955) used baker's yeast as a source of  $\beta$ -glucosidase. Baker's yeast is cultivated for commercial purposes on a rich medium (White, 1954); assuming it to be *S. cerevisiae*, this alone would account for its  $\beta$ -glucosidase activity in contrast with Lodder & Kreger-van Rij's results on arbutin plates: the latter authors were using yeast cultivated on less rich media.

It is evident from the foregoing results that any observations on  $\beta$ -glucoside splitting are much affected by the techniques used. With this reservation, some general relationships do emerge from the quantitative estimate of splitting activity:

(i) When grown on a 'maximal' medium, probably all these yeasts can split aesculin to a measurable degree. Table 5 shows that the largest group (39 %) comprises those which split aesculin and salicin but not arbutin. This percentage might, however, be very different for different groups of yeast. And, of course, changing their nutritional status would give a different response pattern. For instance, a suspension which was negative for aesculin when grown on A2G agar, was also negative for salicin or cellobiose, though in five out of the thirty-eight cases the suspension split arbutin. Yeasts grown on MYGPA never split arbutin only.

(ii) In agreement with the findings of Stelling-Dekker (1931) it may be said that *Hansenula* strains are very strong splitters and *Pichia* strains weak. The active splitting by the strains of *Rhodotorula* studied agrees with the data of Lodder & Kreger-van Rij, but the results with members of the genus *Kloeckera* do not.

(iii) *Candida* and *Saccharomyces* strains varied a great deal in their splitting capacities. The weak *Saccharomyces* included many osmophilic strains, formerly called *Zygosaccharomyces*. *Saccharomyces mellis* and *S. baillii* are very weakly active groups; and also *S. rouxii* (in particular the former *Zygosaccharomyces nussbaumeri*). In the species *S. pastori*, the strains which had been named *Z. pastori* were very weak splitters, whilst the one representative of *Z. pini* was very strong. This difference cuts across their classification as the same species, thus agreeing with the observations of Wickerham (1952), Kudriavzev (1954) and Barnett & Ingram (1955). *S. lactis* and *S. florentinus* were the two most active  $\beta$ -glucoside splitters amongst Lodder & Kreger-van Rij's species of *Saccharomyces*.

If it be held, as hitherto (cf. Lodder & Kreger-van Rij, 1954), that tests with different  $\beta$ -glucosides are completely interchangeable, then the genus *Kloeckera* already provides an interesting anomaly: Lodder & Kreger-van Rij (1952) said it was arbutin-negative; Wiles (1953) described *K. apiculata* as aesculin-negative but salicin- and cellobiose-positive (Table 10) and Ingram (1955) stated that the apiculate yeasts utilize cellobiose. The work described in the present paper does indicate that *Kloeckera* species are active  $\beta$ -glucoside splitters. So much so that the demonstration by Lowings (1956) that a strain of *K. apiculata* was capable of collapsing the structure of picked strawberries led the present writers to investigate possible cellulase activity in Lowing's



strain (LTS 5); the results, however, were negative. The only indication from previous workers that a strain might be able to split one  $\beta$ -glucoside and not another was that quoted above for *K. apiculata* (Wiles, 1953). The results given in Table 2 suggest that yeasts do vary in this way but that it would have been wrong to draw this conclusion from Wiles's results. As Table 2 shows, *K. apiculata*, including Wiles's T70 strain, is a strong aesculin-splitter. Perhaps his negative results were because the aesculin growth test was insufficiently sensitive to detect the splitting.

For a full understanding of the  $\beta$ -glucoside splitting capacities of yeasts it is necessary to know much more about their enzymic basis. Veibel (1950) suggested that  $\beta$ -glucosidase acts irrespective of the nature of the aglycone, though the rate of reaction may vary a great deal. But it appears difficult to explain the results described in the present paper on the basis that all yeasts produce only one  $\beta$ -glucosidase with different quantitative potentialities for each substrate, because the relative potentialities are so different for different yeasts. Thus, for instance, though *Candida tropicalis* (NCYC 4) was a strong salicin-splitter, it was not possible to detect any arbutinase activity at all with this strain. In contrast, several strains of *Saccharomyces cerevisiae* did not split salicin so strongly but gave positive results with arbutin (see MYGPA results in Table 2). Also, the results discussed previously for *Pichia membranaefaciens* grown on different breakdowns of MYGPA suggest that more than one enzyme is present. It cannot be certain from such data that yeasts do produce more than one  $\beta$ -glucosidase, because the cell surface is selective and differences might arise in the transport of the substrates into the cell. It should, however, be easy to avoid this uncertainty by experiments with enzyme preparations. A cell-free extract of baker's yeast, the active part of which was thermolabile and non-dialysable, was found capable of splitting aesculin (see Brunet & Kent, 1955); and an appreciable degree of extracellular  $\beta$ -glucosidase activity was present in the suspensions (see Table 7). In view of this, the data just quoted have an added significance. At one time it was thought that there was only one  $\alpha$ -glucosidase in yeasts (see Ingram, 1955). The study of yeast  $\beta$ -glucosidase seems likely to reveal that it, too, includes a number of distinct enzymes (cf. Jermyn, 1952).

It should be clear from this discussion that the data given in this paper are unlikely to throw much light on the classification of yeasts, except iconoclastically. For example, before deciding that *Torulopsis holmii* does not split salicin (see Table 2) it is necessary to think not only in terms of the yeast's nutrition (the growth medium), but also quantitatively; how many organisms split how much glucoside during what period? The quantitative interpretation of differences held previously to be qualitative is always likely to confuse distinctions used in classification (Mayr, 1942).

It is possible to answer, at least partially, the questions asked in the introduction. Within the limits imposed by the sensitivity of the methods for detecting aglycones it appears that any one yeast able to split one  $\beta$ -glucoside will not necessarily split all the others. Thus when grown on A2G agar *Pichia polymorpha* NCYC 56 split all the  $\beta$ -glucosides tested except arbutin, whereas

*Saccharomyces delbrueckii* var. *mongolicus* CBS 705 split aesculin only, and *S. cerevisiae* NCYC 353 split arbutin only. As already pointed out, the ability to split the  $\beta$ -glucosides is dependent markedly on the kind of medium on which the organism is grown. Even when grown on the complex medium, however, there are still many yeasts which do not split arbutin, salicin or cellobiose, though all are perhaps capable of splitting aesculin. It may be suggested with some confidence therefore that yeasts vary in the  $\beta$ -glucosidases they contain and that some yeasts are not capable of synthesizing certain enzymes, notably arbutinase, even under very favourable conditions of growth.

The factors which influence the differences in the rate of splitting have not been examined in sufficient detail to decide whether these rates are of value for classification. It is known (e.g. Veibel, 1950) that, besides the influence of pH and temperature (not studied here), the activity of  $\beta$ -glucosidases is dependent on the concentration of glucoside, and is usually decreased in the presence of glucose, aglycone and of certain inhibitors such as heavy metal ions. It was found in the present work (cf. Fig. 4) that splitting was arrested at a degree, sometimes far short of completion, characteristic for the different strains. Because the only major variable was the increase in aglycone concentration, probably this was responsible. Thus it might be possible to classify yeasts by measuring their ability to split glucosides in the presence of different amounts of aglycone, but such a technique would require rigid control of the size of the inoculum and other conditions and may seem impracticable.

It would seem desirable that for purposes of classification yeasts should be grown on the richest possible medium so that their full potentialities can be developed. For example, taxonomists have abandoned most of the nitrogen-assimilation tests (contrast Lodder, 1934, with Lodder & Kregger-van Rij, 1952) following Wickerham's (1946) demonstration that many yeasts, previously said to be unable to grow on  $(\text{NH}_3)_2\text{SO}_4$ , asparagine or urea as N-sources, could in fact do so when they had an adequate supply of vitamins. It is difficult, however, to decide what constitutes a maximal medium. For example, *Pichia membranaefaciens* shows salicinase but no aesculinase or arbutinase activity when grown on nutrient agar (Table 6). On all other media this yeast showed varying activities against aesculin and arbutin but not against salicin. It seems likely therefore that even on an optimum medium not all yeasts will hydrolyse arbutin, salicin or cellobiose. It would be worth while to utilize all these three compounds for classification purposes, but it is obviously important to carry out quantitative estimation of the degree of splitting. Further work, however, on the rates of hydrolysis and the effect of inhibitors (including aglycones), will be necessary before a satisfactory rationale can be fixed for using these tests for purposes of classification.

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## The Typing of *Escherichia coli* by Bacteriophage: its Application in the Study of the *E. coli* Population of the Intestinal Tract of Healthy Calves and of Calves Suffering from White Scours

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**SUMMARY:** A bacteriophage method of classifying strains of *Escherichia coli* which inhabit the alimentary tract of cattle has been evolved. It was possible to divide the strains into a large number of types by this method. Varying proportions of strains isolated from the faeces of human beings, sheep, pigs and poultry were also typable by the phages employed. This method of phage typing was used to study the origin and behaviour of the *E. coli* population in the alimentary tract of healthy cows and calves, and of calves suffering from white scours, in one self-contained herd (herd A) and to a lesser extent in twenty-seven other herds. Several types of *E. coli* were often found in the same faecal specimen in cases of scouring, as well as in healthy calves. Seventy different phage types were found in the faeces of the healthy calves in herd A, many occurring infrequently. Thirty-two different types were discovered in the faeces of the scouring calves in this herd; only one strain of one type was found in scouring calves that was not found also in healthy calves. Two types were found commonly in the calves but rarely in the cows. The examination of strains of *E. coli* isolated from faeces of cows and calves at daily and weekly intervals indicated that some types usually remained dominant for a week or so and were then gradually succeeded in dominance by other types. In some animals frequent and sudden changes were apparent; in others one type might be the only type isolated from the faeces for a number of weeks continuously. Changes of dominant phage-type commonly occurred in faeces of calves during the time they were suffering from white scours. The mother did not appear to be a frequent source from which a calf acquired its *E. coli*; the calf pens themselves seemed a more probable source. Exceedingly large numbers of *E. coli* were found in the faeces of healthy and scouring calves during the first 14 days of life, a period when white scours occurs; very much smaller numbers were found in older animals. Studies on material from the twenty-seven other herds supported the findings in herd A; many phage types of *E. coli* were found in these herds that had not been found in herd A. The aetiology of calf scours is discussed in the light of these and other observations.

*Escherichia coli* is well recognized as having an important association with serious intestinal disorders of infants, calves and possibly the young of other species of animals. It is not intended to review the literature on the association of *E. coli* with white scours in calves since this has been ably carried out by Lovell (1955). It is noteworthy, however, that considerable advances have been made recently in the study of this disease, particularly by Lovell and his colleagues. These workers have elucidated the mechanism of the protective action of colostrum and have shown that the K antigens of *E. coli* and their antibodies are associated with protection from the clinical disease. Attention was also paid to the epidemiology of the disease (see also Wood, 1955). The

calves used in these investigations by Lovell and colleagues were obtained and brought to one centre before they had consumed any colostrum. Most of them were then given colostrum deprived of one or more of its natural components. Consequently, we decided to study the epidemiology of the disease under normal farm conditions. The present paper deals with such a study on a self-contained commercial herd (A), special attention being paid to the origin and behaviour of the *E. coli* population of the alimentary tract of healthy calves and cows, as well as of calves suffering from white scours. Less intensive examinations were also conducted on calves from twenty-seven other herds.

We decided to evolve a bacteriophage method for classifying the strains of *Escherichia coli* we isolated from calves because it represented a new approach to a problem on which all the available epidemiological evidence is of a serological nature, and because it has been successfully employed in the study of several diseases caused by other bacteria. Earlier work (Nicolle, LeMinor, Buttiaux & Ducrest, 1952), too, had shown that *E. coli* from human beings could be classified by means of phages.

## METHODS

### *Phage typing*

*The isolation of phages.* A search for suitable phages was first made by modifications of the cross-culture technique (Fisk, 1942). The results were not satisfactory and the search was continued by using the more classical method of examining specimens of sewage and faeces. Such specimens were incubated in Difco broth at various temperatures between 20 and 37° for 6–24 hr., sometimes with the addition of cultures of *Escherichia coli* hitherto regarded as untypable. They were then centrifuged at 3000 r.p.m. for 45 min. and the supernatant fluids either heated to 58° for 30 min. or treated with chloroform (Fredericq, 1950). These fluids were spotted on to plates of nutrient agar previously spread with different broth cultures of strains of *E. coli*. The plates were incubated at 28° for 18 hr. Phage action was revealed by zones of complete or semicomplete lysis, or by discrete plaques. Activity due to colicines only was confirmed by inability to transmit; such preparations were discarded.

*Purification and propagation of phages.* Lytic areas were picked from the plates referred to above and mixed, in different dilutions, with a broth culture of the susceptible strain of *Escherichia coli*, spread over a plate of nutrient agar and incubated at 28°. A discrete plaque was picked as soon as plaques were distinctly visible and replated with the propagating strain. This process was repeated twice. Finally, a discrete plaque with some of the surrounding bacterial growth was picked into broth and incubated at 28° until lysis occurred. More susceptible bacterial culture was then added and the process repeated until a high-titre phage preparation was judged to have been obtained. Any bacteria present were then killed by heating to 58° for 30 min. Occasionally this procedure also destroyed the phage content, so preparations of heat-sensitive phage were sterilized of bacteria by shaking with a few drops of chloroform (Fredericq, 1950).

*Titration of phage preparations.* Tenfold dilutions of the phage preparations were made in phosphate buffer ( $\text{KH}_2\text{PO}_4$ , 3.4 g.;  $\text{Na}_2\text{HPO}_4$ , 6 g.; distilled water, 1000 ml.) A broth culture of the susceptible strain of *Escherichia coli* was spread evenly over the surface of an agar plate, allowed to dry, and then spotted with one drop ( $\frac{1}{150}$  ml.) of each dilution of the phage preparation. Plates were incubated at  $28^\circ$  for 18 hr. and then read. The highest dilution which produced a large number of plaques that were semi-confluent or nearly so was chosen as the critical test dilution, i.e. that suitable for routine typing tests. Although it is customary in most methods of phage typing to select the highest dilution which produces confluent lysis as the critical test dilution, it was deliberately decided not to do this in the present studies since a few of the preparations inevitably contained colicines as well as phage particles; the critical test dilution adopted prevented any resulting confusion.

*Method of setting-up tests.* A modification of the method devised by Wilson & Atkinson (1945) for the phage typing of *Staphylococcus aureus* was employed. Nutrient agar plates were dried at  $37^\circ$  for 2 hr. with their lids partly open. Four drops (0.08 ml.) of an 18 hr. broth culture of the strain of *Escherichia coli* to be typed were spread evenly over the surface of one of the nutrient agar plates by means of a glass spreader. When these had dried, the phage preparations in their critical test dilutions were spotted on to each plate by means of a dropping pipette (150 drops/ml.). For the sake of convenience, the bottom of each plate was marked with a grease pencil into a number of squares equal to the number of phage preparations in use. When the drops had been absorbed the plates were incubated at  $28^\circ$  for 18 hr. and read. Many phage preparations were discarded because tests carried out with a selection of strains of *E. coli* showed that they had an identical action. Finally, a group of 16 different phages, A, B, C, D, E, G, H, L, M, O, T, X,  $Z_1$ ,  $Z_2$ ,  $Z_3$  and  $Z_4$ , were chosen for routine use in typing strains of bovine origin. Another 8 phages,  $Z_5$ – $Z_{12}$ , were developed for typing strains isolated from other species of animals.

In preliminary studies, with a rich nutrient agar medium and incubation at  $37^\circ$ , some of the phages even in high concentrations occasionally exhibited no lytic action on some of the strains of *Escherichia coli* which were known to be susceptible to them. Attempts were made to overcome this by using different media and by using the cultures diluted; the latter had the undesirable effect of enhancing colicine action. The present method, particularly with the incubation temperature of  $28^\circ$ , was much more satisfactory. Many strains were retyped by this method several times during a period of  $1\frac{1}{2}$  years and, apart from the variations inherent in any method of phage typing that depends on the pattern reactions given by strains with a number of test phages, they showed no significant variation in their susceptibility.

*The isolation and identification of strains of Escherichia coli.* Deep rectal swabs were taken and cultured on to plates of MacConkey agar which were incubated at  $37^\circ$  for 24 hr. A number of colonies, usually 10, from each plate were subcultured separately into tubes of nutrient broth and of MacConkey broth. The latter were incubated at  $44^\circ$  in a water bath for 24 hr. Cultures which produced acid and gas in this medium were considered to be *Escherichia coli*



faecal type 1 and the corresponding cultures in nutrient broth, which in the meantime had been incubating at 37°, were immediately available for phage typing; the remaining cultures were discarded.

*Nutrient agar.* The nutrient agar used was prepared by solidifying Difco Nutrient Broth no. B3 with 1.5 % (w/v) New Zealand agar.

#### *Epidemiological studies*

*Herd A.* This dairy herd, in which autumn and winter calving was practised, consisted of approximately 65 Shorthorn cows and their followers. It was self-contained, no cows or calves having been purchased in the last 6 years, and all the cows were machine-milked. The indoor accommodation consisted of one long byre at the end of which were ten pens, separated from each other by low wooden partitions, in which all the calves spent their first 3–4 months of life. The number of calves kept in each pen was mainly determined by their total number. When a cow was observed to be in the early stages of labour she was moved to an empty pen where she remained until 24 hr. after the calf was born. The calf remained in that pen for a few days only and was transferred to another with other calves of approximately the same age. All calves were given their mothers' colostrum, usually by bucket, for the first 4 days. They were fed bulk milk twice daily for the next month, when the feeding with milk substitutes began.

White scours had occurred in the calves in this herd for the last few years and all cases had responded to treatment with streptomycin. It was agreed that, should any cases occur during the present investigation, streptomycin therapy should be withheld until the last possible moment compatible with saving the calf's life, and that in the meantime the diet of milk should be replaced by one of milk and water or water only, in the hope that this would render the disease less acute and provide us with a longer period during which observations could be made. From time to time it was possible to introduce some variations in the procedures in this herd. They will be referred to when necessary.

Between August 1954 and April 1955, 60 calves were born and 16 of them developed white scours. Four of 25 calves born between August and November, 4 of 23 calves born between November and January and 8 of 12 calves born between January and April developed white scours. Much less information was available about the other herds studied.

### RESULTS

#### *The distribution of different phage types of Escherichia coli in cows and calves in herd A and other herds*

The main results of phage typing 1365 cultures from 63 healthy cows, 2500 cultures from 36 healthy calves and 622 cultures from 16 scouring calves in herd A and 237 cultures from 49 healthy calves and 531 cultures from 82 scouring calves in 27 other herds are shown in Table 1. The types listed are only those that formed not less than 1 % of the total number of cultures examined from at

least one of the five sources. Phages  $Z_3$  and  $Z_4$  are not included in Table 1 since none of the cultures upon which they were active belonged to this category. The cultures from herd A were obtained from August 1954 to April 1955. During this time the faeces of some of the calves and cows were examined every day for several days and others were only examined once. Consequently, the frequency distribution of the different phage types may not be accurate. The cultures from the other herds were obtained between February and July 1955. None of these calves was examined more than once.

*Herd A.* The total number of different phage types found in the faeces of the healthy cows, healthy calves and scouring calves in herd A was 69, 70 and 32 respectively. Since many of the types were found only very infrequently, it is probable that the comparatively smaller number of cultures examined from scouring calves accounted for the smaller number of types found in them. Only 22 types from the cows contained 1.0 % or more of the total number of strains examined from this source and, including the untypable strains, these types together formed 82.7 % of the whole. The corresponding figures for the healthy calves and scouring calves in herd A were 22 and 87.0 % and 20 and 97.3 % respectively. Of all the cultures examined from scouring calves, it is noteworthy that only one culture was of a type not also found in cultures from the faeces of healthy calves. Only 18 % of the cultures from the latter belonged to types that were not also found in the scouring calves. Most of the phage types present in the calves were found also in the cows and vice versa, although there were some variations between the frequency distribution of the phage types in the two groups that were probably significant. Phage type 2, for example, the most common type isolated from the faeces of healthy and scouring calves forming 16.0 and 16.7 % of the whole respectively, comprised only 0.7 % of the strains from cows; this type was also quite uncommon in the faeces of calves from the other herds. Again, phage type 22 comprised 8.1 % of the strains from healthy calves in herd A but was never isolated from the cows. These types, however, were rather exceptional.

*Other herds.* The number of phage types found in the faeces of the healthy calves and scouring calves in these herds was 46 and 65 respectively. There were 21 types found in the healthy calves that were not found in the scouring calves and 38 types found in the latter that were not found in the former. The proportion of cultures from scouring calves which belonged to types found also in healthy calves was 53 %; the corresponding figure for healthy calves was 63 %. These differences between scouring and healthy calves were much greater than those which existed in herd A. This was not surprising since the cultures came from 27 herds in all, only a relatively small number originating from each herd, these sometimes having been isolated from scouring calves only or healthy calves only. No one type found in the scouring calves from these herds comprised more than 7.5 % of all the cultures examined, indicating that, taking the herds as a whole, no predominant phage type was associated with scours. Many different phage types were found in the calves in these herds that had not been found in herd A. For example, as far as scouring calves were concerned, 49 % of the cultures examined from these herds belonged to 50 phage types

which had not been found in herd A; and 30 % of the cultures from herd A belonged to 17 types not found in these herds. A much higher proportion of cultures from these herds was untypable than was the case with those from herd A, a fact that was not surprising since the phages had been developed for use in this herd.

*The phage typing of strains of Escherichia coli from other animals*

A small number of strains of *Escherichia coli* from animals other than cattle was phage typed to determine whether the method might be of any use in studying the distribution of *E. coli* in these animals.

*Human beings.* Of 50 strains isolated from the faeces of 50 people, only 17 were typable by means of the 16 phages used for typing the bovine strains. A further 5 strains were typed by the use of another 8 phages, Z<sub>5</sub>-Z<sub>12</sub>. Seven of the types had also been found in the faeces of cattle. Not more than 5 strains belonged to the same phage types.

*Pigs.* Of 50 strains isolated from the faeces of 50 pigs maintained on farm A, 23 were lysed by one or more of the phages of the 'bovine' group; of these, 13 belonged to types which had been found in either the cows or the calves. A further 10 were typable by means of the Z<sub>5</sub>-Z<sub>12</sub> phages. Not more than 3 strains belonged to any one type.

A similar number of cultures from pigs kept on c. 50 different farms were examined. Of these, 30 were acted upon by phages of the 'bovine' group, 22 of which belonged to types which had been found in the farm A cattle. A further 6 strains were typable by means of the phages Z<sub>5</sub>-Z<sub>12</sub>. No one type comprised more than 3 of the 50 strains.

*Sheep.* All except 6 of 50 strains isolated from the faeces of 50 sheep, few of which came from the same flock, were lysed by the 'bovine' group of phages; thirty-eight belonged to types which had been found in cattle. A further two strains were acted upon by the additional phages. No one type contained more than 4 strains.

*Poultry.* Of 50 strains of *Escherichia coli* isolated from the faeces of 50 chickens that belonged to different flocks, 35 were phage-typed by the 'bovine' group of phages. Of these, 22 belonged to types that had been found in cattle. A further 10 strains were typable by the Z<sub>5</sub>-Z<sub>12</sub> group. No more than 3 strains fell into one type.

*The distribution of different phage-types amongst 150 cultures  
of Escherichia coli isolated from the same faecal specimen*

The number of different phage types of *Escherichia coli* found by examining 150 colonies from single specimens of faeces obtained from 10 different healthy calves or cows belonging to herd A was studied. The same loopful of faeces was cultured on 4 plates of MacConkey agar to ensure that 150 well-separated colonies could be picked in all. The number of types present in each specimen varied from one to eight, the exact grouping of the cultures in each specimen according to phage type being 150; 149, 1; 101, 49; 146, 3, 1; 125, 21, 2, 2;





[illegible]

**+ = lysis.**

\* The types listed in this table are only those that formed no less than 1% of the total number of cultures examined from at least one of the five sources. Phages  $Z_3$   $Z_4$  are not included in the table as none of the cultures upon which they acted fell into this category.

89, 38, 13, 6, 4; 86, 45, 11, 5, 3; 60, 60, 27, 1, 1, 1; and 120, 10, 10, 3, 3, 2, 1, 1. As will be seen later, when 10 or even fewer colonies were examined from each specimen of faeces it was usual to find at least 2 types to be present. This multiplicity of types existing in the same animal was by no means found to be confined to the bovine species. The number of types found amongst 10 colonies from each of 10 faecal specimens from pigs varied from 2 to 8, the average being 5.3. The corresponding figures for sheep were 1 to 6 and 2.8, for chickens 1 to 2 and 1.6 and for human beings 1 to 7 and 2.8.

*The phage types of Escherichia coli present in the faeces of 25 cows in herd A at each of six consecutive weekly examinations*

Four colonies of *Escherichia coli* picked from MacConkey plates inoculated with individual faeces samples from 25 cows in herd A each week for 6 weeks were phage typed; the results for 3 cows are illustrated in Table 2. The number of different phage types present amongst the 4 colonies obtained from any single faecal specimen varied from 1 to 4, the average being 1.7. The total

Table 2. *The phage types of Escherichia coli present in the faeces of 3 cows in herd A at 6 consecutive weekly examinations*

Cow no.	Phage types present* in 4 colonies of <i>E. coli</i> on the following consecutive weeks					
	1	2	3	4	5	6
1	101 (1)	101 (3)	101 (2)	101 (4)	101 (4)	101 (3)
	80 (2)	82 (1)	45 (2)			3 (1)
	UT (1)					
2	31 (4)	142 (4)	142 (4)	142 (3)	142 (3)	8 (4)
				45 (1)	8 (1)	
3	UT (4)	87 (4)	24 (4)	105 (4)	80 (4)	8 (4)

\* The number of each phage type present is given in parentheses.

UT=untypable.

number of phage types found in the 600 cultures examined was 51, 65 % belonging to one or other of 10 types; 13 types were found only once. The most common type found was type 101, forming 16 % of the total cultures examined. The number of different phage types found in each cow over the 6-week period varied from 3 to 10, the average being 6.2. In some cows the same phage type was present in the faeces at most of the weekly examinations (cow no. 1). Other types were also present but they did not usually persist from week to week (cow no. 2). In other cows one phage-type only was found at several weekly examinations, this type being succeeded in predominance by another type (cow no. 2). A more unusual result was to find a different phage type predominating at every examination (cow no. 3). Despite the fact that the cows were kept in very close proximity to each other and ate the same food there was no evidence to indicate that changes in the *Escherichia coli* population of any cow from week to week was accompanied by similar changes in any of the other cows.

*The relationship between the phage types of Escherichia coli  
found in cows and in their calves*

The results of typing 10 colonies of *Escherichia coli* from the faeces of 24 cows and 10 colonies from their calves, both series being obtained when the calves were 4 days old, is shown in Table 3. All the calves spent the first day of life with their mother. The calves of pairs 1-10 were then given their mother's milk by bucket for the next 4 days; the calves of pairs 11-24 suckled their mothers for these four days, the mother being brought to the calf pen twice daily for this purpose. Even when untypable strains were considered identical,

Table 3. *The relationship between the types of Escherichia coli  
in the faeces of mothers and their calves at 4 days old*

Pair no.	No. of types present in		No. of types common to calf and mother
	Calf	Mother	
1	1	2	0
2	4	2	2
3	2	5	0
4	2	4	0
5	4	2	1
6	1	1	0
7	1	2	1
8	3	5	2
9	3	2	0
10	1	2	0
11	3	3	2
12	1	1	1
13	2	5	2
14	3	1	0
15	2	1	0
16	2	5	0
17	4	2	0
18	1	3	0
19	3	2	0
20	5	2	1
21	1	2	1
22	4	4	1
23	2	1	0
24	1	2	0

Ten colonies were examined from each faecal specimen

evidence of some of the phage types of *E. coli* in the faeces of the calf being also present in the faeces of its mother was only found in 10 of the 24 cases; in 6 cases, one type was common to calf and mother and, in a further 4 cases, two types. In pair no. 12 all the colonies examined from mother and calf belonged to phage type 2 indicating that, at least, in this pair the calf had probably acquired its *E. coli* population from its mother. It should be noted in these comparisons that from 1 to 5 types were found at one examination in both cows and calves, the averages being 2.3 and 2.1 respectively, a fact which emphasizes the very limited spread of *E. coli* from mother to off-spring that was occurring.

In a further experiment, 10 colonies of *Escherichia coli* isolated at each of 8 weekly examinations from the faeces of each of 6 calves and their 2 foster mothers were phage typed. The calves were kept as 2 groups of three in separate pens, A and B, to which the foster mothers were brought twice daily for suckling. Each group always suckled the same foster mother. At only 3 of the 8 weekly examinations was a phage type found in one or more of the calves in pen A that was also found at the same time in the foster mother. The corresponding figure for the calves in pen B and their foster mother was 2. Eleven different phage types were found in all in the faeces of the foster mother of calves in pen A, 5 of which were also found at one time or another in the faeces of her calves. It is significant that 7 of these 11 types were also found in the faeces of calves of pen B, indicating very strongly that the foster mother was probably not the most likely source of the *E. coli* population of her calves. Eight phage types were found in the faeces of the foster mother of the calves in pen B, of which 4 were also found in the faeces of her calves, not one of them being found on more than two occasions. The phage types found most frequently in the calves of pen B were never found in their foster mother. The phage types present in the calves in any pen were much more closely related to each other than to those of the foster mother. Phage type 22 formed 33 % of the cultures examined from the calves in pen B. It was not discovered in either of the foster mothers or in the calves in pen A.

*The phage types of Escherichia coli present in the faeces of 8 healthy calves in herd A at weekly examinations during the first 9 weeks of life.*

Ten colonies of *Escherichia coli*, isolated each week for 9 weeks from the faeces of two groups of 4 calves kept in separate pens, C and D, and fed the same bulk milk, were phage typed. Of the total of 72 faecal specimens examined, 20 yielded colonies all of a similar type, 27 of 2 types, 12 of 3 types, 8 of 4 types and 5 of 5 types, the average being 2.3. During the 9-week-period, 8 to 16 different types were found at one time or another in the faeces of each calf, the average being 12.5. Of the total of 90 cultures examined from each calf the most common type in each individual comprised 17–55 of the cultures, the average being 25. The number of different phage types discovered in the 720 cultures examined from all the calves was 37, 24 being present in the calves of pen C and 29 in pen D. The 16 types that were common to both pens comprised 94.5 % of the total number of cultures examined from the calves in pen C and 85 % of those from pen D.

As in the case of the cows examined at weekly intervals, considerable differences were noted between individual calves. Two calves in pen C, for example, yielded cultures predominantly consisting of phage type 2 for 3 weeks, this type being succeeded by other types for 3 weeks, and then being replaced in predominance by phage type 2 again in the final 2–3 weeks. By contrast, the faeces of another calf in this pen yielded a number of types, not one of which was predominant on more than 1 week.

Comparing the results for pens C and D, some types were more commonly found in one group than in the other. For example, 98 cultures from the



calves in pen C belonged to phage type 2, and 8 to phage type 22; the corresponding figures for pen D were 16 and 56, respectively. When the results of each weekly examination were compared, it was noted that the phage types present in the calves in the same pen generally bore more relationship to each other than they did to those from the calves in the other pen. However, the *Escherichia coli* present in the faeces of some individual calves bore no relationship at all to those of the other calves examined at the same time. Some phage types made a relatively sudden appearance in the faeces of a number of calves in the same pen at the same time and continued to be found for varying periods afterwards. For example, phage type 37 was the predominant phage type in the calves of pen C at the fourth weekly examination; it had never been discovered in them at the previous examinations and was not found in the calves in pen D. Again, phage type 22 suddenly appeared in 3 of the 4 calves in pen D on the seventh week and was found commonly in them at subsequent examinations. It was also found in one of the pen C calves in the seventh week but not subsequently.

*The phage typing of Escherichia coli isolated from the faeces of healthy and scouring calves at daily examinations*

The results of phage typing 10 cultures of *Escherichia coli* obtained every day for 12 days from the faeces of 13 calves, 8 of which developed white scours during this period, is illustrated in Table 4. The first examination was performed at the end of the first day after birth. A further 7 cases in which examinations had not been made before scouring were also studied; the results were similar to those shown in Table 4. Unless stated, no calf was treated with streptomycin. The number of different phage types in the faeces of the healthy and scouring calves at each examination of 10 colonies varied from 1 to 5. The average was 2.0 for the healthy calves, one type only being found in 38 % of the examinations. The corresponding figures for scouring calves was 2.1 and 37 % during the scouring period and 2.4 and 22 % during the non-scouring period. No one phage type was found to persist in the faeces of any of the healthy calves throughout the 12 daily examinations, although type 71 was found in the faeces of calf 3 on all except the fifth day, being the major type present on 4 days. The general finding was that one type predominated for a few days, and then was gradually succeeded by another type which might in turn be succeeded by another, although on many days, 3, 4 or even 5 different types were found amongst the 10 colonies examined. The number of different phage types found in the faeces of each calf during the 12 days varied from 3 to 13, the average being 8.

The picture was essentially the same in scouring calves as in healthy calves, this being particularly noticeable in calf 13 in which the scouring period was long. In this calf, phage type 96 was predominant in the early scouring period, being gradually superseded in predominance by phage type 2, and then by phage type 71 which was finally gradually replaced by phage type 80, the disease process then being arrested by the oral administration of streptomycin. In many cases, the phage type most commonly found during the

Table 4. *The phage types of Escherichia coli present in the faeces of healthy and scouring calves in herd A in the first 12 days of life*

Calf no.	Phage types* present in 10 colonies of <i>E. coli</i> isolated on the following days after birth											
	1	2	3	4	5	6	7	8	9	10	11	12
<b>Healthy calves</b>												
1	2 (10)	2 (10)	2 (10)	2 (9) 59 (1)	2 (8) 4 (1) 60 (1)	2 (10)	38 (10)	38 (6) 4 (3) 3 (1)	4 (1) 3 (9)	3 (10)	3 (4) 77 (6)	3 (3) 77 (7)
2	3 (1) 38 (1) 64 (4) 65 (4) 65 (4)	4 (3) 38 (2) 65 (4) 69 (1)	2 (6) 36 (2) 69 (2)	74 (9) 88 (1)	74 (8) 3 (2)	74 (3) 3 (7)	74 (2) 3 (2) 77 (6)	3 (10)	3 (8) 74 (2)	3 (6) 77 (4)	3 (10)	77 (10)
3	71 (5) 36 (5)	71 (2) 2 (3) 3 (3) 101 (2)	71 (1) 2 (7) 64 (2)	71 (6) 2 (3) 3 (1)	1 (1) 22 (2) 36 (4) 63 (1) 83 (2)	71 (9) 1 (1)	71 (2) 64 (2) 63 (6)	71 (2) 63 (4) 2 (4)	71 (2) 2 (8)	71 (4) 2 (4) 63 (1) 60 (1)	71 (2) 2 (6) 80 (2) 38 (1)	71 (3) 2 (4) 80 (2) 38 (1)
4	88 (3) 83 (7)	88 (7) 30 (3)	88 (6) 83 (2) UT (2)	88 (3) 45 (4) 39 (3)	88 (2) 83 (1) 45 (6) UT (1)	45 (10)	45 (10)	45 (10)	45 (10)	45 (10)	45 (10)	45 (10)
5	101 (5) UT (3) 72 (2)	101 (5) UT (5)	UT (10)	UT (10)	UT (10)	UT (10)	UT (6) 4 (4)	UT (6) 4 (4)	UT (8) 4 (2)	4 (10)	4 (10)	4 (10)
<b>Scouring calves</b>												
6	2 (10)	71 (6) UT (4)	71 (5) 60 (2) 24 (3)	60 (4) 2 (6)	60 (3) 2 (6) 71 (1) ++ ++	60 (6) 2 (4) ++ ++	60 (2) 2 (4) 71 (4) ++ 2 (10)	60 (10) 2 (4) 71 (4) ++ 2 (10)	60 (7) 2 (1) 71 (1) UT (1) 2 (10)	60 (2) 2 (6) 63 (2)	63 (2) 36 (8)	36 (10)
7	3 (10)	3 (8) 2 (2)	2 (9) 60 (1) ++ ++ ++	2 (10) ++ ++ ++	2 (9) 3 (1) ++ ++ ++	2 (10) ++ ++	2 (10) 2 (9) 3 (1)	2 (10)	2 (10)	2 (10)	2 (1) 38 (9)	2 (1) 38 (9)

8	2 (4) 130 (5) 60 (1)	2 (2) 130 (3) 36 (2) 72 (2) 47 (1)	2 (7) 130 (1) 36 (1) 77 (1)	2 (10) +	2 (9) 60 (1) +	2 (6) 60 (3) 130 (1) +	2 (5) 130 (1) 60 (1) 72 (2) 88 (1)	2 (5) 130 (4) 3 (1)	2 (3) 130 (7)	47 (9) 101 (1)	47 (6) 3 (4)	47 (8) 3 (2)
9	65 (5) 71 (4) 26 (1)	65 (1) 3 (2) 38 (6) 102 (1)	3 (3) 38 (2) 64 (3) UT (2)	3 (7) 38 (2) 64 (1)	3 (3) 58 (7) +	3 (2) 58 (7) 10 (1) +	3 (1) 38 (6) 65 (3) +	65 (10) +	65 (10)	65 (10) +	65 (3) 3 (3) 77 (3) 31 (1) +	65 (1) 77 (6) 38 (2) 2 (1)
10	3 (5) 101 (1) 112 (3) 128 (1)	112 (1) 128 (2) 2 (6) 143 (1)	11 (4) 31 (6)	11 (8) 31 (2)	11 (8) 31 (2)	128 (10)	128 (9) 88 (1)	128 (10)	128 (10)	128 (10)	40 (9) 32 (1)	Died
11	113 (6) 145 (2) UT (2)	113 (2) 145 (5) UT (3)	2 (10) 48 (2) 101 (4)	2 (4) 48 (2) 101 (4)	48 (2) 101 (5) 38 (2) UT (1) +	48 (5) UT (1) 2 (2) 143 (2) +	48 (4) 2 (4) 45 (1) 113 (1) +	48 (5) 2 (2) 45 (2) 143 (1) +	48 (8) 143 (2)	48 (8) 143 (2) +	48 (7) 2 (3) +	48 (1) 2 (7) 143 (2)
12	36 (9) UT (1)	36 (5) 26 (5)	26 (8) UT (2)	26 (9) UT (1)	26 (10) 26 (3) UT (7)	26 (3) UT (7)	26 (3) UT (7)	UT (10) 118 (1)	UT (9) 118 (1)	UT (10) +	UT (10) +	UT (10)
13	144 (10)	144 (4) 2 (1) 88 (3) 80 (2)	2 (4) 88 (1) 60 (5)	2 (4) 96 (6)	96 (8) 60 (2)	96 (5) 2 (3) 60 (2)	96 (6) 2 (2) 60 (1) 3 (1)	2 (1) 3 (9)	2 (5) 3 (4) 96 (1)	2 (4) 3 (3) 60 (3) +	2 (4) 3 (4) 60 (1) 131 (1) +	2 (7) 60 (1) 80 (1) 43 (1) +
Days	13	14	15	16	17	18	19	20	21	+	+	+
13 (cont.)	2 (10)	2 (2) 80 (4) 3 (3) 71 (3)	2 (4) 3 (3) 71 (3)	71 (9) 80 (1)	71 (10)	71 (9) 80 (1)	71 (2) 80 (8)	80 (10)	80 (10)	80 (10)	80 (10)	80 (10)
	+	+	+	+	+	+	+	+	+	+	+	+

\* The number of colonies belonging to each phage type is given in parentheses.

The degree of severity of disease condition of the scouring calves is indicated by the number of + signs. The absence of a sign indicated that the faeces were normal.

scouring period was present in the faeces for a number of days before scouring began and invariably afterwards. Sometimes there was an interval in the pre-scouring period in which that phage type was not found in the faeces. For example, phage type 2 was the only type discovered in the faeces of calf 6 at the end of the first day after birth; it was not present at the second and third day but reappeared when scouring commenced on the fourth day, and was not found at all on the final day of scouring. Further examples of this are seen in the case of phage type 65 and calf 9 and phage type 128 and calf 10. During the scouring period, 34 types were found in all in the 15 scouring calves examined, 12 of the types occurring reasonably frequently. The number of different phage types in each calf during the scouring period varied from 1 to 8, the average being 4. No one particular phage type was found to be present in more than 6 of the 15 cases and in only 4 of these was it present in considerable numbers. This type (phage type 2) was also found in the faeces of 3 of the 5 healthy calves in this experiment, being present in considerable numbers in 2 of them. In all observations (Table 1) it was discovered to be the most frequent type found in healthy and scouring calves in herd A. The next most common type in the present experiment was phage type 71 which was found in 5 scouring calves, being present in considerable number in 3 of them. It is noteworthy that phage type 71 was also found in healthy calf 3 at all except one of the twelve examinations. Phage types 71 and 2 were very commonly found in the faeces of calves in the first half of the calving season, when most of the calves were born and when the incidence of white scours was low.

*The possibility of bacteriophages influencing the distribution  
of different phage types in the intestinal contents of calves*

It was foreseen that qualitative changes in the *Escherichia coli* population of the faeces of scouring and healthy calves might be brought about by the selective action of bacteriophages present in the intestinal tract. Consequently, many of the faecal specimens examined in the previous experiment were retained in the refrigerator until the phage typing results were available. When a change in predominating type occurred in the faeces of a calf the faeces collected from this calf on the previous day and on the same day as the change occurred were examined for the presence of phage that was lytic for the phage type of *Escherichia coli* previously predominant. For example, in the case of calf no. 1 (Table 4), the faeces collected on the sixth and seventh days were examined for phages active on phage type 2. Over forty examinations of this kind were conducted; only in two were positive results obtained, the amount of phage present in those two being so small as to make very remote the possibility that phage had significantly influenced the number of the *E. coli* on which they were active. A more remote possibility, at least in short-term studies, was that change of phage type might be induced by phage in the intestinal tract bringing about lysis of the predominant strain with the emergence of phage-resistant variants of the predominating strain, as distinct from permitting other strains to occupy the dominant position. If such variants influenced the typing results obtained with the routine test phages, they would



be acted upon by fewer of the test phages that acted upon the strain from which they were derived, and not at all by the other test phages. A comparison of the phage reactions of the coli types found in the same calf at successive examinations yielded no positive evidence of this having occurred.

*The number of Escherichia coli present in the faeces of healthy and scouring calves in herd A*

The approximate numbers of viable *Escherichia coli* present in the faeces of 8 healthy calves at different times after birth is illustrated in Table 5. Very high counts (c.  $10^9$  bacteria/g. faeces) were observed at 2 days after birth. Counts remained high for 14 days, after which they decreased until at 50 days they were quite low. Very low counts were also observed at 150 days after birth.

Table 5. *The number of Escherichia coli present in the faeces of healthy calves at different times after birth*

Calf no.	No. $\times 10^4$ of viable <i>E. coli</i> /g. of faeces at the following days after birth					
	2	4	7	14	50	150
21	120,000	12,000	20,000	6,000	1	10
22	250,000	10,000	100,000	6,000	150	2
23	50,000	2,000	40,000	30,000	5	30
24	200,000	7,000	60,000	50,000	3	500
25	200,000	7,000	60,000	50,000	3	50
26	600,000	40,000	50,000	10,000	3	10
27	50,000	10,000	25,000	600	1500	100
28	40,000	1,200	5,000	3,000	10	50

As compared with faeces from young healthy calves, low counts of *Escherichia coli* were obtained from the faeces of cows, e.g. the approximate numbers in the faeces of 10 cows were 80, 50, 30, 25, 5, 5, 5, 3, 1 and  $0.3 \times 10^4$ /g., respectively.

The numbers of *Escherichia coli* present in the faeces of scouring calves were similar to those for the healthy calves shown in Table 5. The numbers present, in calves scouring at 4 days of age were 7,500, 9,000, 120,000  $36,000 \times 10^4$ /g. faeces, and in calves scouring at 7 days of age were 60,000, 40,000, 200,000 and  $25,000 \times 10^4$ /g. faeces. Counts carried out on the faeces of calves that developed white scours were also made before, during and immediately after the scouring period. There was no significant difference between the results for each of these three periods when each calf was considered individually.

*The phage types of Escherichia coli in the faeces of calves during outbreaks of white scours in different herds*

The distribution of different phage types among 5 colonies of *Escherichia coli* isolated from the faeces of scouring calves during outbreaks of white scours in 27 herds other than herd A was also studied. All the calves from each herd were scouring at the same time, the time when the faecal samples were examined. It was also possible to examine faecal samples from healthy calves

in herds where scouring calves were present. The history of these herds was much less complete than that of herd A. Most of the herds were not self-contained, some calves being purchased in the market.

The number of phage types present amongst 5 colonies of *Escherichia coli* isolated at each examination of the faeces of healthy and scouring calves varied from 1 to 5. The average for faecal specimens from 61 scouring calves at each examination was 1.7, one phage type only being found in the faeces of 28 calves. The corresponding figures for 45 healthy calves was 1.9 and 16 respectively.

Although one or two phage types of *Escherichia coli* were found more frequently in one herd than in another, there was no evidence to indicate that one type only was associated with white scours in any particular herd at any particular time. It is noteworthy that in two herds different phage types were found in the faeces of each calf. As in the case of herd A, the phage types present in the scouring calves were also found not infrequently in the faeces of healthy in-contact calves.

#### DISCUSSION

The fact that many more phage types of *Escherichia coli* were identified in herd A alone by means of phage typing than has been found by other workers who have approached the study of *E. coli* infection in calves by serological methods is not surprising since Nicolle *et al.* (1952), working with strains of *E. coli* of human origin, found that several different phage types could be found amongst strains of the same serological type. A possible explanation of this is that many of the phage types differ from each other by means of acquired phage resistance only, i.e. some strains through contact with different phages in the field have become resistant to them and as a result their susceptibility to the test phages may have altered; such strains would then accordingly be differentiated from otherwise identical strains which had been exposed to other and different phages. Positive evidence exists that such changes take place in other species of bacteria and is responsible for some type differentiation (Smith, 1948; 1951 *a, b*; Felix & Anderson, 1951; Anderson & Felix, 1953; Tee, 1955). Since faeces are the natural habitat of *E. coli* and are also a good source of phages active against these organisms it is not difficult to imagine these phages having an impact on phage type differentiation. Such changes would probably occur more infrequently and more slowly than *in vitro* experiments indicate and, consequently, they would not be a source of confusion in short-term studies. This method of typing probably classified together strains with a similar history of phage infection and at the same time may differentiate from each other strains essentially the same except for a dissimilar history of phage infection. Its use, therefore, is probably restricted to short-term epidemiological investigations.

Up to eight phage types were found during the examination of 150 colonies of *Escherichia coli* obtained from the same specimen of faeces, some of the types only occurring once or twice. This, considered with the fact that each gram of calf faeces may contain  $10^9$  *E. coli* indicates the magnitude of the task

of studying the qualitative aspects of the *E. coli* population of the alimentary tract. It is obviously only possible by present methods to obtain information on the dominant type or types. Many more types may be present in quite considerable numbers, their presence being masked by the overwhelming numbers of the dominant types. However, the present studies have thrown some light on the behaviour of *E. coli* in the calf alimentary tract. It is apparent that the alimentary tract of each calf has to be considered as a separate entity since the *E. coli* flora of some individual calves occasionally bore no resemblance to that of other calves kept in the same pen and given the same milk for as long as 9 weeks. In general, however, a much greater degree of similarity existed between the flora of calves kept in the same pen than between calves kept in different pens but given the same bulk milk. The phage typing results on such groups of calves tends to indicate that the pens themselves may be the most likely source of origin of the *E. coli* found in the calves. In view of the almost constant habit of calves of sucking animate and inanimate objects it is not difficult to visualize this possibility. The mother did not appear to be a potent source of origin of the *E. coli* population of the alimentary tract of her calf. It is conceivable, though, that the phage types found in some of the calves may also have been present in the faeces of the mother in undetectable numbers.

The change of the predominating phage types which occur in the intestinal tract is interesting. The same phage type remained dominant in some calves and cows for many weeks and frequent and relatively sudden changes took place in others; the usual picture was of a gradual change of predominating type during 1-2 weeks, thus resembling in many respects what happens in man (Sears, Brownlee & Uchiyama, 1949; Sears & Brownlee, 1951).

Qualitatively and quantitatively, the behaviour of *Escherichia coli* in the alimentary tract of scouring calves so closely resembled that of the healthy calves that one might wonder what part the *E. coli* played in the disease process of the scouring calves. The association of these bacteria with white scours has, however, now been well established (see Lovell, 1955). Fresh evidence was obtained from chemotherapeutic studies on many of the scouring calves in the present investigation. For example, ever since herd A had been established, the oral administration of streptomycin had had a very significant effect in controlling white scours. One dose usually resulted in a complete cessation of diarrhoea, the faeces being of normal consistency on the following and succeeding days and the number of *E. coli* present being almost negligible. Only one treated calf died during the present investigation, this calf having been left too long before being treated. The evidence from the other herds is even more convincing (Smith & Crabb, 1956). In these the *E. coli* in the faeces of scouring calves before and after treatment were tested for sensitivity to chemotherapeutic agents. When they were sensitive to streptomycin, therapy with this agent was almost invariably successful, but when they were resistant, it was of no avail; treatment with other agents to which the *E. coli* in the faeces were sensitive yielded satisfactory results. There can be little doubt, therefore, that *E. coli* played an important role in the cases of white scours studied in the present investigation. Considering all the evidence it is apparent that less



emphasis should be placed on regarding white scours as a disease associated with a relatively few specific types of *E. coli*. It would be advisable to regard the development of the disease in some calves in a herd as an adverse result of the endeavour of the calf to establish an equilibrium with the types of *E. coli* that have colonized its alimentary tract as suggested by Lovell (1955), the present studies stressing that the actual phage types of *E. coli* present in the alimentary tract are of less importance than the resistance of the calf. This view does not preclude the possibility that there may be herds in which specific strains are mainly responsible for the scours. It merely indicates that scours may still occur in these herds in the absence of the specific strains.

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## Abortive Transduction of Motility in Salmonella; a Non-replicated Gene Transmitted through Many Generations to a Single Descendant

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**SUMMARY:** Cultures of non-motile *Salmonella* strains treated with phage lysates of motile strains produce in semi-solid agar: (i) swarms, each consisting of a clone of motile bacteria, attributed to complete transduction of motility; (ii) trails, i.e. unbranched linear groups of microcolonies stretching some millimetres from the site of inoculation, attributed to abortive transduction of motility; the latter was thought to result from the importation by phage of a gene conferring motility, which was not replicated but was transmitted, as a supernumerary gene, down one line of descent (unilinearly) to but one of the descendants of the organism which received it, the trail marking the path of the gene-bearing descendants of successive generations.

The present work has led to a modified hypothesis: that the phage-imported non-replicated gene confers on the organism the ability to synthesize motility conferring (MC) particles, which are distributed amongst its non-gene-bearing progeny, which cannot make new ones; that each particle may be unilinearly transmitted for many generations; and that while one MC particle confers motility in broth, several are needed to enable a bacterium to travel in a semi-solid medium.

The main evidence for this hypothesis is provided by pedigrees of bacteria made motile by abortive transduction, isolated by micromanipulation from treated suspensions. All such bacteria produce mainly non-motile offspring. A minority (exceptional or *E* bacteria) produce clones which after 10-15 generations include 20 to 100 motile descendants; the rest give clones containing only a few (0-12) motile organisms. *E* bacteria are identified as ones containing the supernumerary gene, the others as ones motile through possession of a few MC particles only.

In each of six extensive pedigrees a single *E* bacterium was isolated from amongst the 9th-21st generation progeny of the original *E* parent. None of a large number of other motile bacteria isolated in collateral sublines (and so *ex hypothesi* not gene-bearing) produced more than 12 motile descendants. These data show that the *E* character (ability to produce 15 or more motile descendants) is unilinearly transmitted.

Any motile bacteria found amongst the progeny of a non-*E* bacterium after six or more generations were inferred to be motile through possession of one MC particle each; of many such which were isolated none produced more than one motile descendant; when this in turn was isolated, a single motile organism was sometimes again detected in the clone produced. This shows that there is unilinear transmission of motility (in broth).

Samples of populations of motile bacteria isolated by micro-manipulation from treated suspensions were transferred to semisolid agar, or to individual droplets; the proportion of bacteria which generated trails was about equal to the proportion found to be *E*. This was to be expected if both characters reflect the presence of the non-replicated gene.

The MC particle is probably a flagellum, or a granule which determines the production of one. The particle presumed to account for the unilinear transmission of the *E* and trail-forming characters is most economically interpreted as a phage-imported fragment of genetic material which has failed to replace its homologue in the genetic organelle of the recipient organism.

Many different hereditary properties may be transferred from one *Salmonella* strain to another by applying phage lysates of one strain to a culture of the other; this transfer of hereditary characters (transduction) is believed to result from the transfer by phage of genes or fragments of genetic material from organisms of the lysed strain to organisms of the recipient strain (Zinder & Lederberg, 1952; Stocker, Zinder & Lederberg, 1953; Lederberg & Edwards, 1953; etc.). Transduction of characters is inferred from the detection of a clone showing the transferred character, and organisms of such clones transmit the new character to all their descendants. The inheritance of the new character by all the progeny of a transformed organism and the total disappearance of the alternative character which was present before treatment are striking features, both of the transduction of characters in *Salmonella* and of the analogous phenomenon of transformation of hereditary characters in pneumococcus, etc., mediated by the application of deoxyribonucleic acid (DNA) extracted from an appropriate strain. In both systems a proposed explanation is that extraneous genetic material regulating the new character has entered the recipient organism and replaced the homologous genetic material which determined the old character, the latter material being lost. There is evidence for linkage of some of the transferable genetic factors, which suggests that these factors normally form part of a differentiated genetic organelle, perhaps analogous to a chromosome; if extraneous genetic material became incorporated into such a structure it would presumably be thereafter replicated and transmitted to the progeny by whatever mechanisms effect the replication and transmission of the organism's intrinsic genetic material.

In the course of experiments on the transduction of motility from motile (wild-type) to non-motile *Salmonella* strains (Stocker *et al.* 1953) a phenomenon was encountered which suggested that some bacteria which became motile by transduction produced non-motile progeny. Such unstably transformed bacteria were ascribed to 'abortive transduction', in contrast to complete transduction which produces stably transformed ones. Its occurrence was inferred from the behaviour of certain non-motile strains when incubated on a semi-solid medium. Untreated cultures produced surface growth, strictly confined to the site of inoculation. Cultures treated with a suitable lysate produced: (i) spreading swarms, each swarm being the motile progeny of a completely transformed organism; (ii) groups of micro-colonies in the depths of the medium, the colonies of each group being arranged along, or close to, an unbranched line extending several millimetres from the site of inoculation. These groups were called 'trails'. The microcolonies comprising a trail yielded on subculture only non-motile growth, similar to that of the parent strain; yet their situation showed that each must have grown from a parent which had moved through the semi-solid medium. It was suggested that each trail arose from a bacterium made motile by abortive transduction; and that when this divided it produced one non-motile daughter, which formed a micro-colony *in situ*, and one motile daughter which travelled on through the semi-solid medium, and itself repeated the process when it divided, and so on at each successive division.

Such abortive transduction of motility was tentatively ascribed to introduction by phage into a non-motile recipient of a normal or wild-type gene which failed to displace from the genetic organelle its homologue determining non-motility, but which caused the recipient organism to become motile despite the simultaneous presence of this homologue; and it was suggested that such a supernumerary gene would not be replicated, so that at division it would pass to only one of the daughters. In this model an organism having a certain character transmits this character (and the factor or particle determining it) down a single line of descent to but one of its progeny in any generation; this will be called 'unilinear transmission'. Fig. 1 illustrates the original hypothesis that trail formation results from the unilinear transmission of motility amongst the progeny of a bacterium abortively transformed to motility. This paper describes experiments designed to test this hypothesis, by direct observation of the motility of the descendants of motile bacteria isolated by micromanipulation from lysate-treated suspensions. Professor J. Lederberg (University of Wisconsin) has also investigated this problem by micromanipulation techniques, and I have had the benefit of frequent discussion and reports of the progress of his work. However, our materials, experimental results and conclusions differ to some extent and we are therefore reporting them separately (see Lederberg, 1956).

The nature, and indeed the existence, of the non-replicated determinant postulated to account for abortive transduction is uncertain, but for brevity it will be referred to as a 'supernumerary gene' and the organism presumed to contain it will be called the 'gene-bearing' organism; the justification for this description will be discussed later.

It soon became evident that the hypothesis in its simple form could not account for the results obtained. In the first place, counts of colonies in trails indicated that during the formation of a trail several micro-colonies were initiated per generation time, instead of the one predicted by the original theory. Secondly, single motile rods isolated by micro-manipulation from phage-treated cultures were found to produce, after many generations, progeny comprising a large number of non-motile together with several motile bacteria, instead of the single motile descendant predicted by the original theory. These discrepancies might be explained by 'phenotypic lag'. Normal (wild-type) strains of *Salmonella* are motile by means of flagella, of which each bacterium has several. The non-motile strains used in the present experiments lack flagella, which is presumably why they are non-motile. A bacterium which becomes motile by abortive transduction must owe its motility to an acquired ability to produce one or more flagella. When it divides, the daughter which does not receive the supernumerary gene perhaps receives some part of the locomotor apparatus of the parent, e.g. some of its flagella or some of the enzymes concerned in their synthesis, so that this daughter is motile, at least for a time. From what is known of phenotypic lag in other situations it might be supposed that an organism which was thus motile only because it contained 'products of gene action' would produce descendants whose motility would decrease at each successive generation, until finally all were non-motile. As will be shown,



micromanipulation experiments gave results incompatible with this supposition, but explicable by the following hypothesis.

*Hypothesis of motility conferring particles, transmitted unilinearly amongst progeny.* This hypothesis supposes that the presence of the supernumerary gene in an organism causes it to produce stable particles, which are distributed amongst the daughters at division, and that the presence of even a single

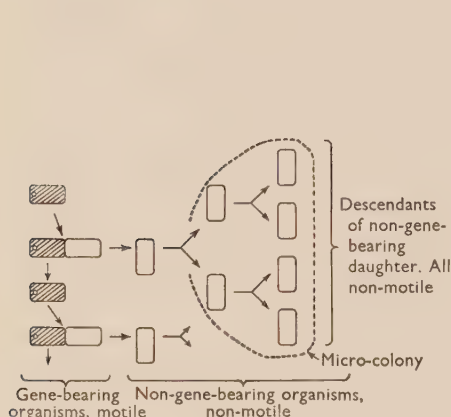


Fig. 1

Fig. 1. Original hypothesis of trail formation; unilinear transmission of supernumerary gene which confers motility. Each division of gene-bearing organism produces (i) a gene-bearing daughter which moves on, (ii) a non-gene-bearing daughter which is non-motile and produces a micro-colony of the trail. One micro-colony initiated at each generation. Only one motile descendant of gene-bearing organism. ○, supernumerary gene; ▨, a motile organism; □, a non-motile organism.

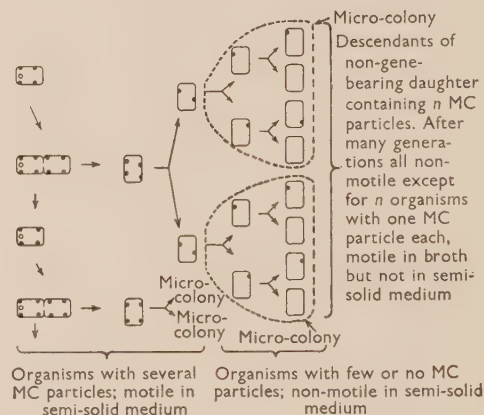


Fig. 2

Fig. 2. Final hypothesis of trail formation; unilinear transmission of supernumerary gene which confers ability to generate motility-conferring (MC) particles, which are themselves unilinearly transmitted. Each division of gene-bearing organism produces (i) a gene-bearing daughter (with several MC particles) which moves on, (ii) a non-gene-bearing daughter which contains several MC particles (4, in this diagram) and in consequence moves through the semi-solid medium; after some generations the number of MC particles/organism amongst its descendants falls below some critical level (2, in this diagram) so that each ceases to move, and initiates a micro-colony of the trail. Several micro-colonies (2, in this diagram) initiated at each generation. Unilinear transmission of each MC particle, and so of 'motility in broth'; gene-bearing organism produces many descendants motile in liquid (though not in semi-solid) medium. ○, supernumerary gene; ●, motility-conferring particle.

particle in a bacterium confers on it some degree of motility. The progeny of a motile but non-gene-bearing organism will then include after a time several descendants, each containing a single motility-conferring particle; each such organism will transmit its particle, and hence a degree of motility, to one of its descendants. The hypothesis thus postulates two orders of unilinear transmission, the primary line of transmission being that of the supernumerary gene, whose presence in an organism results in the production of motility conferring particles, each of which is transmitted down a secondary line. To account for the absence of branching in trails it is necessary to postulate that



only bacteria which contain several particles can travel through semi-solid agar, though a bacterium with one particle is motile in broth. This hypothesis, and the way it accounts for trail formation, is illustrated in Fig. 2. The motility conferring particles are probably flagella, or granules each of which determines the production of a flagellum, but as this is unproven, they will be referred to only as motility conferring (MC) particles.

#### METHODS

*Organisms.* The phage used and most of the bacterial strains were those of Stocker *et al.* (1953). SW 543, an O strain of *Salmonella paratyphi-B*, was the parent strain of sublines: SL 160, used in a few of my experiments; and SW 666, used by Lederberg (1956). For the present purpose both sublines may be regarded as identical with strain SW 543. SW 966 is a *Salmonella* O strain of group B (strain 3010-49 of Dr P. R. Edwards), identified as *S. paratyphi-B* by Lederberg (personal communication). Most of the micro-manipulation experiments were made on strain SW 541, a *S. typhimurium* O strain which has never been observed to mutate spontaneously to motility, and which yields large numbers of trails (and swarms) when treated with a suitable lysate. Strain TM2, a motile *S. typhimurium*, was generally used as donor strain. In experiments on *S. paratyphi-B* strain SL 160 lysates of a motile derivative of this strain were used; such lysates are more effective than *S. typhimurium* lysates (Lederberg, personal communication). In all cases the lysates were made with phage PLT 22.

*Treatment of non-motile cultures with phage lysates.* One volume of a broth culture of a non-motile strain was mixed with an equal volume of a bacteriologically sterile lysate with a phage titre of at least  $3 \times 10^8$  particles/ml., and the mixture was incubated at 37°. For observations on trails, samples of this mixture were plated after 30 min. on to a semi-solid gelatin agar medium (Stocker *et al.* 1953), and the plates were incubated for 12-18 hr. at 37°. To avoid the trails being obscured the mixture was so diluted that most plates did not develop any swarms. For determining the number of micro-colonies arising in a trail per hr. of incubation at 37°, the plates were incubated for about 12 hr. at 37°, and then held at room temperature overnight. This second period allowed the minute micro-colonies at the distal end of the trail to grow to a visible size; the solidification of the gelatin-containing medium prevented the initiation of any new colonies by motile bacteria.

For the isolation of motile bacteria by micromanipulation, the mixture of bacteria and phage was incubated for 90-120 min. at 37° before transfer to the micromanipulation chamber. In some experiments many of the bacteria isolated failed to grow; this mortality was largely avoided by using broth cultures near the end of the log phase in place of fully grown cultures, and by diluting the phage-treated culture with broth at intervals during the period of incubation. The results of micromanipulation experiments were generally similar, whether log-phase or overnight cultures were used.

*Micro-manipulation.* A Singer micromanipulator (Barer & Saunders-Singer,

1948) was used. Angled micropipettes were made by the dropping-weight method (de Fonbrune, 1949). Micromanipulation methods generally were based on those of de Fonbrune (1949). Drops of broth were deposited on the lower surface of a coverslip which formed the roof of a chamber filled with liquid paraffin. It was not convenient to use a fresh micropipette for each operation; it was found that when only one rod was taken up at a time, the sterility of the pipette could be assumed when a single rod (of about the same size) had been expelled. Observations were made by low-power darkground microscopy, with an 8 mm. objective of N.A. 0.5 and a long-working-distance Cooke, Troughton and Simms phase-contrast condenser unit, with the annulus intended for the 2 mm. objective.

For isolation of motile bacteria, a drop of phage-treated culture was placed at one point of the coverslip, and droplets of sterile broth at others. Motile rods frequently travelled round the periphery of the drop, whereas the non-motile ones tended to sediment into the concavity of the drop, leaving the border free, so that it was generally easy to pick up single motile rods from the edge of the drop, even when they were present in a proportion of 1:10<sup>4</sup> or less. This operation was facilitated by the use of a 'trapping drop', that is a drop of sterile broth placed so that it just came into contact with the drop containing the phage-treated culture. Motile bacteria swam out into this sterile broth, and could be isolated very easily.

Motile rods thus isolated were transferred to the previously prepared broth droplets. The oil-chamber was kept at room temperature, and re-examined at intervals; and the number of motile and non-motile rods in each droplet was estimated and recorded. Rods which were spinning or twitching, movements which were obviously not Brownian, were recorded as motile. Even when a droplet contained very large numbers of non-motile bacteria ones in translational movement were detected with surprising ease, partly because of their tendency to travel round the periphery of the drop (Pl. 1, figs. 3, 4). When more than about eight motile rods were present in a drop it was difficult to count them exactly, except by removing them one by one; counts recorded as greater than eight are therefore for the most part estimates. In the pedigree experiments motile bacteria amongst the progeny were re-isolated from the droplets to fresh droplets, the large numbers needed being set out in grid formation by the use of the mechanical stage; it was found necessary to interrupt periods of observations at room temperature by refrigerating the oil chamber at c. 4° for up to 24 hr., but this did not seem to affect the subsequent behaviour of the organisms.

#### OBSERVATIONS ON TRAILS

The majority of about 20 non-motile *Salmonella* strains tested produced trails as well as swarms when treated with lysates; the ratio of trails to swarms varied from about 1:1 to 100:1 or more. Stocker *et al.* (1953) found that a non-motile strain treated with a lysate of some other non-motile strain commonly produced swarms, which was taken to indicate non-homology of the genetic factors causing non-motility in the two strains; many such combina-

tions yielded trails also. In contrast, treatment of a culture of a non-motile strain with a lysate of that same strain never evoked trails (or swarms). Some non-motile strains when grown on a semi-solid medium spontaneously produce microcolonies just below the inoculated surface of the medium (Stocker *et al.* 1953); but these spontaneous satellite microcolonies were never arranged linearly, like the microcolonies of a trail.

In most strains the trails were short, and did not increase in length when incubation was continued for longer than 18 hr. The spontaneous termination of a trail presumably indicates that after a certain number of generations there is no longer present, amongst the descendants, a viable organism containing the supernumerary gene. However, a non-flagellated strain of *Salmonella paratyphi-B*, SW 966, produced trails which after 42 hr. of incubation at 37° were about 20 mm. long (Pl. 1, Figs. 1, 2). No branching was seen, even in these long trails. They seemed always to extend laterally, more or less directly away from the site of inoculation, which suggests a negative chemotactic response to some product of bacterial metabolism.

Counts of the number of colonies initiated per hour of incubation were made by the method described above. Lysate-treated cultures of *Salmonella typhimurium* strain SW 541 incubated at 37° for 15 hr. produced trails made up of 80–90 colonies. Its generation time at this temperature is 20–30 min., so at most 45 generations could have elapsed during this time. Thus an average of at least two colonies were initiated at each division of the gene-bearing organism; this indicates that phenotypic delay in loss of ability to move through gelatin-agar lasts between one and two generations at least. The colonies forming a trail were arranged in pairs and small groups, which became confluent as they enlarged; the calculated minimum of two colonies per generation is therefore probably an underestimate.

On two occasions a swarm appeared to have arisen at the distal end of a trail several mm. long; but the strains concerned were known to undergo spontaneous mutation to motility at fairly high rates. From the rarity of such appearances it is inferred that a transformation which is not completed within the first few generations is seldom or never completed thereafter.

#### ISOLATION OF MOTILE ORGANISMS FROM PHAGE-TREATED SUSPENSIONS

Motile bacteria were isolated by micromanipulation, by the method described above, from lysate-treated suspensions of *Salmonella typhimurium* strain SW 541. Most of them became immobilized either at the glass or at the oil interface soon after they had been transferred to individual droplets. This accidental immobilization generally made it impossible to determine motility in the organisms resulting from the first divisions of the originally isolated bacterium. Because of it, counts of actually moving bacteria give only minimum estimates of the number of potentially motile ones present. Droplets each inoculated with a single motile rod were examined at intervals, to ascertain the number of motile and non-motile descendants.



The most informative results were obtained after overnight incubation, by which time each droplet contained some hundreds or thousands of bacteria. At this time the results predicted by the hypothesis of MC particles are as follows (a motile bacterium isolated from a lysate-treated culture will be called an 'initial'). There are three kinds of 'initial':

(i) Immediate descendants of gene-bearing organisms, themselves lacking the supernumerary gene but motile because of the presence of a few (say  $n$  or less) MC particles, obtained from the parent. These particles will be distributed amongst the offspring; after 10 generations the descendants will number  $c. 1000$ , of which only  $n$  or less will be motile, through the presence in each of one MC particle.

(ii) Gene-bearing organisms. An organism of this sort divides to give a gene-bearing daughter, and a non-gene-bearing daughter which contains a few (say an average of  $n$ ) MC particles; the gene-bearing daughter in turn divides in the same way; and the process is repeated in each generation. In consequence after 10 generations there will have been produced in all about  $10n$  particles. As those MC particles produced in the earlier generations will have been distributed, there will be present, in addition to a majority of non-motile descendants, one gene-bearing motile descendant and nearly  $10n$  descendants motile by possession of MC particles.

(iii) Completely transformed organisms, and their descendants. These will produce clones of motile bacteria.

Thus the hypothesis predicts that the populations resulting from  $c. 10$  generations of growth of 'initials' will be of three kinds: (i) mainly non-motile, with a few motile; (ii) mainly non-motile with many motile; (iii) all motile.

In a series of experiments on *Salmonella typhimurium* strain SW 541 (Table 1, Expts. i-vi), growth was obtained from 152 out of 164 'initials' isolated. Five populations of motile rods were inferred to have grown from completely transformed 'initials'. The remaining 147 populations, made up predominantly of non-motile rods, could be divided into two classes with, respectively, few and many motiles, namely: 116 populations with 0-12 motile rods; and 31 populations with 19-80 motile rods. The former class includes 16 populations in which no motile organisms were seen; these are interpreted as populations in which the MC particles responsible for the motility of the 'initial' have escaped detection.

It was at first supposed that populations containing many (e.g. 50) motile rods resulted from the delayed completion of an abortive transformation, resulting in the production of a small clone of fully transformed bacteria. This was not the case, since numerous motile rods isolated from such populations always produced progeny which were nearly all non-motile.

'Initials' producing populations containing many motile rods were always a minority, generally about a fifth of the total. These 'initials' are interpreted as gene-bearing; but as a non-committal label any organism giving rise to more than 15 motile descendants amongst a predominance of non-motile ones will hereafter be termed *E* (exceptional).

In a further series of experiments the immediate progeny of the 'initials',



Table 1. *Populations resulting from overnight incubation of 'initials' isolated from lysate-treated cultures of non-motile strains*

Expt.	Non-motile strain	No growth	Motile clone	No. of 'initials' yielding											No. of initials tested
				Non-motile populations with indicated number of motile organisms											
				0	1	2	3	4-6	7-9	10-12	13-18	19-29	30-80		
(i)	SW 541	1	1	1	2	8	3	0	0	0	6	24			
(ii)*	SW 541	0	1	5	5	2	0	1	0	1	1	19			
(iii)*	SW 541	0	3	1	3	2	1	1	0	1	4	16			
(iv)	SW 541	6	0	12	2	8	2	1	0	3	8	58			
(v)*	SW 541	3	2	2	1	1	3	1	0	0	2	20			
(vi)*	SW 541	2	2	3	4	7	0	1	0	2	3	27			
Sum	SW 541	12	5	24	17	28	9	5	0	7	24	164			
(vii)	SW 544	1	0	1	4	2	4	1	0	2	0	20			
(viii)	SL 160	8	1	2	4	2	2	1	0	2	4	26			
(ix)	SL 160	1	0	2	2	5	2	1	1	0	0	15			
(x)	SW 578	5	0	1	1	1	1	0	0	1	3	14			
(xi)	SW 966	6	1	3	5	3	0	0	2	2	2	30			

SW 541 and SW 544 are non-flagellated strains of *Salmonella typhimurium*, SW 966 is a non-flagellated strain of *S. paratyphi-B* and SW 578 is a 'paralysed' (non-motile flagellated) strain of *S. typhimurium*; these strains were treated with lysates of *S. typhimurium* strain TM 2. SL 160 is a subline of SW 543, a non-flagellated strain of *S. paratyphi-B*; it was treated with lysates of a motile variant obtained from SL 160 by transduction.

\* In these experiments the organisms resulting from the first divisions of some of the 'initials' were separated; the figures shown were obtained by summing those for the sister populations.

when they numbered one to six, were transferred to separate droplets; after overnight incubation the resulting sets of sister populations were examined, and the numbers of motile descendants detected were recorded (Table 2). It will be seen that when the total number of motile descendants of an 'initial' numbered less than 15 they were distributed more or less equally amongst the sister populations of a set; as these 'initials' are interpreted as organisms

Table 2. *Numbers of motile descendants detected in progenies of sister organisms produced by division of 'initials'*

'Initials' isolated from lysate-treated cultures of *Salmonella typhimurium* strain SW 541 were allowed to divide. The resulting organisms, when they numbered 2 to 6, were isolated in separate droplets. After overnight incubation the resulting sister populations were examined; all contained hundreds or thousands of non-motile organisms, as well as the motile ones recorded.

‘Initial’ no.	Sisters separated when they numbered	No. of motile progeny seen in the resulting sister populations							Total no. of motile progeny seen
1	Two	0	0	.	.	.	.	0	
2	Two	0	1	.	.	.	.	1	
3	Two	0	2	.	.	.	.	2	
4	Two	1	2	.	.	.	.	3	
5	Two	1	2	.	.	.	.	3	
6	Two	1	2	.	.	.	.	3	
7	Two	3	6	.	.	.	.	9	
8	Two	5	6	.	.	.	.	11	
9	Two	3	<i>c.</i> 30	.	.	.	.	<i>c.</i> 33	
10	Two	3	<i>c.</i> 40	.	.	.	.	<i>c.</i> 43	
11	Three	*	0	2	.	.	.	2	
12	Three	0	1	8	.	.	.	9	
13	Three	2	3	29	.	.	.	34	
14	Four	*	0	0	2	.	.	2	
15	Four	0	0	1	1	.	.	2	
16	Four	0	1	2	2	.	.	5	
17	Five	2	2	3	5	17	.	29	
18	Six	*	*	0	0	1	2	3	
19	Six	0	0	0	1	1	3	5	
20	Six	0	0	0	3	4	<i>c.</i> 50	<i>c.</i> 57	
21	Six	0	0	1	2	5	<i>c.</i> 65	<i>c.</i> 73	

\* Organism failed to grow.

motile only through the presence of MC particles it is inferred that MC particles are distributed about equally amongst the daughters at division. In the case of *E* 'initials', producing in all more than 15 motile descendants, these were distributed very unevenly, nearly all being found in only one of a set of sister populations. For instance, all but 8 of the c. 73 motile progeny of 'initial' no. 21 were descendants of one of the six organisms resulting from the first divisions of the 'initial'. On the hypothesis adopted 'initial' no. 21 was a gene-bearing organism, and the sister population containing many motile descendants was derived from the single gene-bearing organism of the six into which it divided.

If all the descendants of an organism motile only through presence of products of gene action were non-motile after a period of phenotypic lag of less than 10 generations, then in experiments of this sort motile descendants would be found in no more than one of a set of sister populations, viz. that derived from the gene-bearing sister.

### PEDIGREE EXPERIMENTS

The best evidence obtained for the postulated two different orders of unilinear transmission comes from experiments in which the transmission of the 'super-numerary gene' was followed through many generations. To obtain such pedigrees, a number of motile 'initials' were isolated as before, and the organisms resulting from their first few divisions were separated. Some generations later an attempt was made to select families which had grown from

Table 3. *Summary of pedigrees of six 'initials' isolated from lysate-treated cultures of Salmonella typhimurium strain SW 541*

Pedigree no.	Intermediate isolations of <i>E</i> descendant at generation no.*	No. of motile descendants of		Generations between 1st and last isolation of <i>E</i> organism (g)	Motile descendants per generation (a/g)
		last-isolated <i>E</i> descendant	all its collateral relatives (a)		
1a	4, 10, 13	c. 100	85	20	4.2
3a	2, 3, 9	c. 38	27	9	3.0
10a	2, 3, 4, 13	c. 30	40†	11†	3.5
21y	2, 4, 6	c. 35	28	8	3.5
25a	3, 8, 12, 13	c. 20	55	13	4.2
27a	2, 6, 8, 9	c. 40	29	9	3.2
		Sum	264	70	3.8

\* Generations calculated from estimated population sizes, the 'initial' being taken as generation no. 1.

† The first two generations in pedigree 10a have been omitted in this calculation, because of death of several organisms.

*E* 'initials'; such families were recognized by the presence of a relatively large number (8–20) of motile organisms in the family as a whole; their unequal distribution within a set of sister populations sometimes permitted recognition of one of the set as that likely to contain the gene-bearing descendant. All detectable motile rods were isolated from such droplets, in order, if possible, to re-isolate the gene-bearing organism; this process was repeated at intervals. In some pedigrees motile cells which were inferred to be non-gene-bearing were also isolated. Some experiments of this kind failed, none of the motile descendants isolated after some generations of growth proving to be *E* (i.e. producing more than 15 motile progeny). However, in six pedigrees the transmission of the *E* character, and so by inference of the gene, was followed for 8–20 generations (Table 3).

Three of these pedigrees are set out in Fig. 3. For clarity, only some of the transfers and observations made have been included. The estimated numbers





of motile and non-motile bacteria in a droplet are recorded at each time that bacteria were removed from it. The number of generations elapsing since the isolation of the 'initial' from the treated population has been calculated from the estimated population sizes. For simplicity the final composition for the population of a droplet records the largest number of motile rods known to have been present at any period later than the 7th generation of growth of the organism inoculated into the drop (exclusive of motile organisms removed to other droplets, which are separately indicated). For instance, when a droplet inoculated with one motile rod was later found to contain *c.* 400 non-motile and two motile rods, the result is recorded as 'S + +, 2M', that is 'thousands of non-motiles, two motiles', even if, as sometimes happened, only one, or no, motile rods were detected by the time the population numbered thousands.

*Evidence for unilinear transmission of the E character*

If the *E* character results from the presence of a non-replicated 'gene', then after many generations of multiplication of an *E* 'initial' one *E* descendant would be expected amongst its progeny. In the six pedigree experiments *E* descendants were isolated from amongst the progeny of the (estimated) 9th, 10th, 10th, 14th, 14th, and 21st generations, respectively. All the ancestors of the last-isolated *E* bacterium back to the 'initial' are of necessity *E* also, since their progeny includes the progeny of the final *E* bacterium. If the *E* character is transmitted unilinearly, then in any one pedigree no organism other than the last-isolated *E* bacterium, and its ancestors, can be *E*. In pedigrees 1*a* and 25*a* there were isolated, respectively, more than 50 and more than 30 viable motile bacteria which, by this argument, could not have been *E*. None of the resulting 80 populations contained more than nine motile rods. Similar, though less extensive, results were obtained in the other four pedigrees and in other experiments.

All the ancestors of an *E* organism back to the 'initial' are by definition also *E*; but in the pedigrees some of these ancestors are recognizable as *E*, even without taking into account the motile progeny of the *E* organisms isolated from amongst their offspring. Consider, for instance, organism *b* of generation 13 in pedigree 1*a* (Fig. 3); its progeny included organism *c*, which produced *c.* 100 motile descendants. But even if organism *c* had died its ancestor, *b*, would still have been recognized as *E*, for it produced *c.* 50 other motile descendants. In the same pedigree organism *a* of generation 4 may likewise be recognized as *E* without taking into account its *E* descendant of generation 13 (organism *b*).

On the hypothesis stated, an *E* bacterium divides to produce one *E* daughter, and one non-*E* daughter which will produce a few (< 15) motile progeny. In the six pedigree experiments pairs of sisters of which one proved to be *E* were separated on twelve occasions. All the twelve non-*E* daughters produced some (one to nine) motile progeny.

In pedigree 27*a* (Fig. 3) organism *p*, of the estimated 9th generation, divided to produce two daughters, which had, respectively, 3 and *c.* 40 motile descendants; thus the unique *E* descendant of the 9th generation

behaved just like an *E* 'initial' (Table 2), i.e. divided into dissimilar daughters which produced, respectively, few and many motile descendants amongst a predominantly non-motile progeny.

*Evidence for unilinear transmission of motility-conferring particles*

The bimodal distribution of numbers of motile cells detected in the progeny of 'initials' (Table 1) suggested that the number of MC particles received by the non-*E* daughter of an *E* organism was probably not more than fifteen, and it was inferred that after six generations (when the progeny numbered  $2^6 = 64$ ) or later, the proportion of descendants of such an organism which contained more than one MC particle would be small. Any motile descendant seen at this stage might therefore be assumed to contain only one MC particle, and would therefore be expected to transmit this particle, and hence motility, to only one of its progeny. In the six pedigree experiments 66 viable, motile descendants of the 6th or later generation of sisters of *E* organisms were isolated. All these 66 cells produced populations of non-motile bacteria; and in each of 17 of these populations (26 %) one motile descendant was detected. Similar results were obtained in other experiments, and in no instance has an organism of this kind been observed to produce more than one motile descendant. Accidental immobilization of the bacterium containing the MC particle may account for the non-detection of the expected single motile descendant in the remaining 74 % of the isolations. This high proportion of failures, however, suggested that, even if an organism of the kind under discussion did on occasion produce two motile descendants, this might not be detected. Two further lines of evidence confirm that only one motile descendant is produced.

The more direct evidence is from observations of the actual moment of division of a motile bacterium which was inferred from its pedigree to be motile through possession of a single MC particle. In experiments on *Salmonella typhimurium* strain SW 541 such observations were impracticable, since the bacteria nearly always became immobilized at an interface shortly after transfer to separate droplets. Motile bacteria obtained by transduction from *S. paratyphi-B* strain SL 160 are less liable to accidental immobilization, and in pedigree experiments with this strain the division of a motile bacterium inferred to contain one MC particle was watched on several occasions; in each case one daughter moved on unchecked, while the other was stationary (or in Brownian motion) from the moment of fission.

Further evidence for the unilinear transmission of motility was given by several experiments in which the solitary motile descendant was re-isolated in series. In pedigree 1 *a* (Fig. 3) organism *x* was inferred to be non-gene-bearing, since its 'cousin', organism *a*, was an *E* organism. The descendants of organism *x* when they numbered *c.* 100 included four motile organisms, each presumably motile through possession of a single MC particle. These four were isolated; one of them, organism *y*, produced progeny numbering some hundreds amongst which was detected a single motile organism, which could now certainly be inferred to be motile through the presence of one MC particle only. This organism, *z*, in turn produced a clone of non-motile rods amongst which,

when they numbered thousands, a solitary motile rod was again detected. Such a result seems in itself almost sufficient to establish the occurrence of unilinear transmission of motility and by inference the existence of a unilinearly-transmitted motility-conferring particle.

*The number of MC particles received by a non-E daughter of an E organism*

On the hypothesis adopted, when a gene-bearing organism divides one daughter will receive a few (say an average of  $n$ ) MC particles, and will produce a clone containing  $n$  potentially motile descendants. In the six pedigrees viable rods later recognized as sisters of *E* organisms were isolated on twelve occasions; the number of motile rods detected in the resulting clones varied from one to nine (average 4.4). Since not all potentially motile descendants were detected this gives only a minimum estimate of  $n$ . The counts of numbers of motile descendants depended for the most part on observations made after many generations of multiplication; it seems likely that the probability of thus detecting a potentially motile descendant (i.e. one MC particle) after several generations of unilinear transmission would be about the same as the probability of detecting a motile descendant after several generations of growth of an organism which contained a single MC particle. The latter probability was estimated above as 26%. The true value of  $n$  is therefore estimated as  $4.4 \times 100/26 = 17$ . In this calculation only observations on organisms known to be sisters of *E* organisms have been used.

Other observations in the pedigree experiments can also be used. If it be assumed that the gene-bearing organism grows and divides at the same average rate as the rest of the progeny of an *E* 'initial' it is possible to estimate  $g$ , the number of generations between the isolation of the 'initial' and the last occasion on which its *E* descendant was isolated. A daughter containing an average of  $n$  particles is produced at each generation, so that after  $g$  generations the progeny of the *E* 'initial' will comprise one *E* organism and non-gene-bearing ones containing amongst them about  $n \times g$  particles. Thus, the total number of motile descendants detected in all the collateral lines of a pedigree (i.e. the populations of all droplets except that to which the *E* descendant was finally transferred) divided by  $g$  (the estimated number of generations) will give an estimate of  $n$ . The data for the six pedigrees are given in Table 3; the estimates of  $n$  do not differ much, and their mean, 3.8, is close to the value of 4.4 estimated from sisters of *E* organisms. Adjustment for the estimated proportion of undetected MC particles gives a value of  $3.8 \times 100/26 = 14$  for  $n$ .

#### EVIDENCE THAT ONLY *E* ORGANISMS PRODUCE TRAILS

Trails have been explained above as marking the path through the semisolid medium of the successive bearers of a non-replicated 'gene' which confers ability to synthesize MC particles; and *E* organisms have also been identified as ones containing this supernumerary 'gene'. If this be correct then, of the motile organisms which result from abortive transduction, only the *E* organisms should produce trails. As *E* organisms were recognized in retrospect, by counting their



motile progeny, it was impossible to transfer known *E* organisms to semisolid medium in order to test their trail-forming ability. However, the proportion of *E* organisms was compared with the proportion of trail-forming ones in samples from collections of motile *c* 'initials' evoked by lysates. 'Initials' were picked from a trapping drop and transferred alternately to separate droplets, which were retained in the chamber, or to a larger drop, which, when it had received a number of bacteria, was transferred to a tube of broth. From this tube drops were inoculated on to semisolid medium. Of 57 'initials' kept in the chamber 51 grew and produced predominantly non-motile progeny, and 11 of these 51 (*c.* 22 %) were *E* (20–60 motile descendants each). After overnight incubation at 37°, 21 of 33 plates of gelatin agar, each inoculated with one drop from the tube containing trapped 'initials', gave no growth; it is calculated from the Poisson formula that the 12 drops which did yield growth contained about 15 viable organisms. In the inoculated areas there developed three trails, comprising, respectively, *c.* 35, 60 and 67 colonies. Thus a proportion of 3 in about 15 (*c.* 20 %) of the viable 'initials' from the trapped population produced trails, and 22 % of them were *E*. Pl. 1, fig. 5, shows a trail produced by an 'initial' transferred to semisolid medium. Motile descendants of organisms later diagnosed as sisters (or other collateral relatives) of *E* organisms are by inference not themselves *E*. A number of such motile organisms were transferred from the micromanipulation chamber to gelatin agar medium; none of them produced a trail.

Many 'initials' transferred to semisolid medium produced groups of two, three or four adjacent microcolonies. These 'initials' were presumably non-gene-bearing organisms containing several MC particles, and therefore able to move through the semisolid medium; their immediate progeny would retain this ability until they contained an insufficient number of MC particles. Counts of colonies initiated at each generation during trail formation had similarly indicated that the non-gene-bearing daughter of a gene-bearing organism produced an average of at least two colonies in semisolid medium.

As *E* organisms and their close relatives which contained several MC particles appeared to differ from organisms which contained only one particle by their ability to travel through semisolid medium, other differences in degree of motility were sought. No consistent difference in speed was found, but organisms which contained several MC particles seemed less liable to get stuck at interfaces, and, if they did get stuck, to have a greater chance of breaking away later.

#### PEDIGREES OF 'INITIALS' PRODUCING CLONES OF MOTILE ORGANISMS

##### *Unilinear transmission of motility amongst progeny of sisters of completely transformed organisms*

About 4 % of 'initials' isolated from treated suspensions gave, after overnight incubation, populations containing thousands of motile bacteria which on subculture produced motile populations. This result is attributable to complete



transformation of the 'initial'. When the daughters produced by the first division of an 'initial' had been separated, it was sometimes found that one daughter gave a clone of motile organisms, while the other produced a clone of non-motile ones. This may be explained by assuming that the original bacterium contained two or more identical 'nuclei' or gene-sets and that the phage-imported gene was incorporated into one of these. Thus, when the bacterium divided the daughter which received the transformed gene-set would produce a clone of motile organisms, while the other daughter, which received the untransformed set, would produce a clone of non-motile organisms.

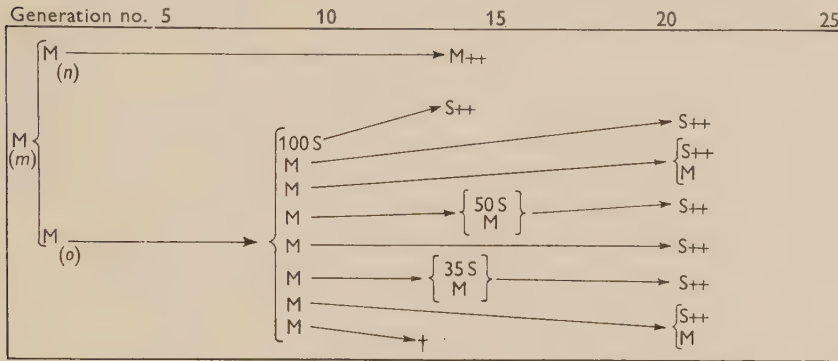


Fig. 4. Pedigree of an 'initial' which produced a clone of motile organisms; the 'initial' (on left of diagram) was isolated from a lysate-treated culture of strain SW 541. Symbols as in Fig. 3.

Lederberg (1956; personal communication) found that when one of a pair of sister populations consisted of motile bacteria, the other, when it consisted mainly of non-motile ones, yet included a few motile organisms, each of which transmitted motility to only one of its descendants. This was observed in experiments on transduction of motility to *Salmonella paratyphi-B* strain SW 666. In experiments on *S. typhimurium* strain SW 541 I obtained similar results. For example, in the pedigree shown in Fig. 4 organism  $n$  produced a motile clone and its sister,  $o$ , produced a clone which consisted of *c.* 100 non-motile and 7 motile descendants. The 7 motile organisms were isolated; of the 6 which grew 4 produced clones in which a single motile descendant was seen at some stage between the 6th and 13th generation. It is inferred that organism  $o$  contained at least 7 MC particles obtained from its parent,  $m$ , which was able to form MC particles because a phage-imported gene had been incorporated into one of its originally identical gene-sets. An alternative explanation would be that motility had been abortively transduced to the 'initial', so that it contained a supernumerary gene and as a result formed MC particles, some of which passed to each daughter when it divided; and that the supernumerary gene later became incorporated into the gene-set in the daughter to which it chanced to pass.

MICROMANIPULATION EXPERIMENTS ON OTHER NON-MOTILE  
SALMONELLA STRAINS

Motile bacteria were isolated by micromanipulation from lysate-treated cultures of two non-flagellated *Salmonella* strains, SW 544 and SW 966, and one non-motile flagellated strain, SW 578; these experiments gave results (Table 1) similar to those described above. Unilinear transmission of 'motility in broth' was observed in pedigree experiments on all these strains.

Experiments on *Salmonella paratyphi-B* subline SL 160 of strain SW 543 gave rather variable results. Sometimes (e.g. Expt. ix of Table 1) the numbers of motile descendants found in the progenies of 'initials' did not show a bimodal distribution. At other times (e.g. Expt. viii of Table 1) the results obtained were similar to those with SW 541. In pedigree experiments it was easy to demonstrate the unilinear transmission through many generations of ability to swim in broth, perhaps because in this strain motile organisms were less prone to get stuck to interfaces. Several attempts were made to obtain pedigrees of *E* organisms in strain SL 160, but mostly without success; none of the re-isolated motile descendants of the 'initial' proving to be *E*. However, in one experiment 19 motile descendants of about the 12th generation were isolated, and one of them showed the *E* character (c. 17 motile descendants).

## DISCUSSION

*The evidence for unilinear transmission of two different characters*

The experiments described were undertaken to test the hypothesis of Stocker *et al.* (1953) that trail formation resulted from the unilinear transmission of motility amongst the progeny of organisms which had been incompletely or 'abortively' transformed. They have led to the conclusions: (i) that trails result from the unilinear transmission not of motility but of the ability to generate locomotor apparatus; (ii) that amongst the progeny of organisms which have generated such locomotor apparatus there is unilinear transmission of ability to travel in a liquid medium, though not in a semisolid one. These two unilinearly-transmitted characters have above been attributed to two hypothetical particles, the 'supernumerary gene' and the 'motility-conferring particle'; though the material basis of the two characters is uncertain this does not affect the validity of the conclusion that two characters are unilinearly transmitted. As this conclusion was unexpected and as the experiments of Lederberg (1956) do not unequivocally confirm it, the experimental data on which it is based have been given in some detail. The argument from the data to the hypothesis may be summarized.

Trails 2 cm. or more long must mark the path through the agar of an organism, or rather of a succession of organisms. If the progeny of the organism which initiated the trail included at any time more than one descendant with the ability to form a trail, the trail would branch; the absence of branching therefore implies unilinear transmission of the character 'ability to produce a trail'.

The pedigree experiments show that, even after 20 generations, amongst the

progeny of an organism made motile by abortive transduction there may be found one descendant, and one only, with the *E* character (ability to produce  $\geq 15$  motile descendants). This indicates unilinear transmission of the *E* character. The pedigree experiments also show that the character 'motility in broth' may be unilinearly transmitted for many generations amongst the progeny of non-*E* motile organisms.

If it be accepted that the pedigrees establish the unilinear transmission of these two characters (*E* character and 'motility in broth') it must be decided whether one or both characters correspond to the unilinearly transmitted 'ability to form a trail'. Several lines of evidence indicate that organisms with unilinearly transmitted 'motility in broth' do not form trails. Bacteria known from their pedigrees to be of this kind did not produce trails when transferred to semisolid medium. Such organisms have been seen to divide and produce one daughter which was non-motile from the moment of fission; whereas it appears from the number of colonies initiated/generation-time in a trail that there is phenotypic delay in loss of motility when a trail-forming organism divides. Furthermore, most motile 'initials' isolated gave rise to several motile descendants which transmitted motility unilinearly, and if each such descendant could form a trail, several trails would start from a common origin; but in fact all or nearly all trails arise singly.

On the other hand, all the data are consistent with the hypothesis that the unilinearly-transmitted *E* character is the same as the unilinearly-transmitted ability to form a trail, and that both reflect unilinearly-transmitted ability to generate locomotor apparatus (MC particles). Parallel tests have shown that 'initials' with the two characters are present in about the same proportion in particular populations.

Lederberg (1956; and personal communication) in experiments with *Salmonella paratyphi-B* strain SW 543, subline SW 666, obtained results which, though not incompatible with the above hypothesis, yet do not unequivocally support it; and in a few experiments on strain SW 543, subline SL 160, I obtained generally similar results. In pedigree experiments on strain SW 543 it is easy to demonstrate unilinear transmission of motility in liquid medium but difficult or impossible to show that the *E* character is transmitted unilinearly. It may be that in this strain the *E* character is nearly always lost within the first few generations of unilinear transmission, e.g. by the loss or decay of the 'gene' presumed to account for it, or through the death of the organism containing it. However, the results obtained with other non-motile strains (Table 1, etc.) suggest that the conclusions as to abortive transduction drawn from experiments on *Salmonella typhimurium* SW 541 are generally applicable.

It has been argued above that only *E* organisms can produce trails in the semisolid medium used. The experiments of Lederberg (1956) clearly show that several organisms amongst the progeny of an 'initial' can produce trails in a semisolid medium with a lower content of agar and gelatin than that of the 'standard' medium used in my experiments. This is not surprising, for in a medium with a sufficiently low gel content a bacterium which contained a single MC particle would presumably be motile, as it is in liquid medium.



*The motility-conferring particle*

If the material basis of the unilinear transmission of 'motility in broth' is a motility-conferring particle, then such particles are presumably concerned in the motility of normal motile *Salmonella* strains also, and unilinear transmission of motility might be expected to occur in other situations where motile bacteria produce non-motile offspring. Mr C. Quadling (unpublished; see Stocker, 1956; Quadling & Stocker, 1956*a*) demonstrated unilinear transmission of motility in two such situations: (i) in some non-flagellated *Salmonella* strains in which he found a very small proportion of motile organisms which were not mutants, since nearly all their progeny were non-motile; (ii) in some strains which were motile when grown at one temperature but on transfer to another temperature no longer produced flagella. Lederberg (1956) found unilinear transmission of motility in *Salmonella dublin* SW 553, another non-flagellated strain in which rare motile organisms occur.

It is generally believed that flagella are the locomotor organs of *Salmonella*, though this is not universally accepted (e.g. Pijper, 1951). This suggests that the MC particle may be a flagellum, or a particle (e.g. basal granule) which determines the production of a flagellum. This would account for the immediate non-motility of the daughter which does not receive the MC particle. On this interpretation salmonellas require several flagella to travel through the semi-solid medium used, though one suffices for motility in broth.

A critical test of the relation between flagellum and MC particle would be to determine, by staining or electron microscopy, the number of flagella on a bacterium inferred from its pedigree to be motile by possession of a single MC particle. Technical difficulties have prevented the performance of this experiment. However, Mr C. Quadling (personal communication) found that in *Salmonella* populations unable to make new flagella there was a good correlation between numbers of flagella seen in stained preparations, and numbers of MC particles inferred from pedigree experiments. This makes it seem probable that the MC particle is a flagellum, or a structure which determines the production of one.

If this be so, the pedigrees of non-*E* organisms imply that the flagella of a dividing bacterium are being distributed about equally between its daughters. This is incompatible with the conclusion of Bisset (1951) who inferred, from electron micrographs and stained preparations, that in dividing Gram-negative bacilli the portion of the cell-wall bearing the parental flagella was retained in its entirety by one daughter, the other daughter growing new flagella. However recent observations (Quadling & Stocker, 1956*b*) on the distribution of numbers of flagella/bacterium in growing cultures in which no new flagella are being formed suggest that, at least in some circumstances, Bisset's conclusion is incorrect for *Salmonella typhimurium*.

It has been calculated above that the non-*E* daughter of an *E* organism probably produces *c.* 15 potentially motile progeny, and therefore probably received from its parent *c.* 15 MC particles; this is considerably more than the average number of flagella/bacterium in a motile derivative of strain SW 541,



as determined by staining the flagella by Leifson's method (1951) (Pl. 1, fig. 6). However, if the MC particle is a granule which secretes a flagellum and if there is some delay between the formation of the granule and its production of a visible flagellum then in a growing culture of a flagellated strain each organism would contain more MC particles than visible flagella, which would account for this discrepancy.

The experiments described above, together with those of Lederberg (1956) and of Mr C. Quadling (unpublished), indicate the occurrence of unilinear transmission of motility in all non-flagellated *Salmonella* strains tested, and also in *Salmonella typhimurium* SW 578, a flagellated but non-motile ('paralysed') strain. It seems that all these strains are non-motile because of a genetically-determined inability of their cells to make new MC particles, despite their ability to utilize and transmit to their progeny any MC particles which they receive from their parents. The genetic factors responsible for non-motility in most of these strains are non-homologous (Stocker *et al.* 1953; Stocker & Quadling, unpublished).

#### *The non-replicated supernumerary gene*

The only circumstance in which trails and, it is inferred, abortive transduction of motility, have been seen is when cultures of non-motile strains have been treated with phage lysates capable also of evoking swarms, i.e. of effecting complete transduction of motility. The first step in abortive transduction is, therefore, as in complete transduction, the bringing-in by a phage particle of something from the donor organism. Trails (and swarms) may be evoked from one non-motile strain by treatment with phage grown on another non-motile strain; therefore the particle transferred in abortive transduction cannot be anything which is present only in motile organisms, and is either a gene or a specific gene product. The unilinearly transmitted particle postulated to account for trail formation may then be either this same particle, or some product of its action, though not necessarily a specific product (Lederberg, 1956). However, the most economical hypothesis is that the particle transferred by phage is a gene, and that this gene itself is subsequently transmitted unilinearly. The best evidence that the particle is a gene would be to show that after unilinear transmission through many generations it sometimes began to multiply *pari passu* with the organisms, i.e. that an abortive transformation became complete. No evidence that this occurs has been met in pedigree experiments, and the evidence that swarms occasionally arise from trails is equivocal. The proof that the unilinearly transmitted particle which confers the *E* and trail-forming characters is a gene, not a product of gene action, is thus incomplete. It might be said that a particle which is not replicated is by definition not a gene and it would perhaps be more precise to say that the particle concerned was a gene in the donor organism, before its transfer to the recipient.

Mr H. Ozeki (personal communication) has recently obtained evidence of abortive transduction of prototrophy to certain purine-dependent mutants of

*Salmonella typhimurium*; his conclusions as to the mechanism are similar to those presented here.

Though the postulated particle has been called a gene, it probably consists of a fragment of a genetic organelle, perhaps a piece of a linear chromosome-like structure. Some evidence for this is given by observations (Lederberg, 1956; Stocker, unpublished) on *Salmonella paratyphi-B* strain SW 543. The gene determining absence of flagella in this strain is linked to a gene which regulates the antigenic character of the flagella (Stocker *et al.* 1953). The immobilizing action of anti-flagellar sera on abortively transformed bacteria suggests that in this strain the non-replicated genetic particle always or nearly always contains both the gene for formation of flagella and the linked gene for flagellar antigenic type; whereas the fragment incorporated into the gene-set in complete transformation frequently contains only the former gene.

The ratio of trails to swarms varies widely from one pair of donor and recipient strains to another; it is not known what determines it. Partial non-homology of the genetic material with that of the recipient might hinder its incorporation into the genetic organelle; but this cannot be the only cause of non-incorporation, for a non-motile strain treated with a lysate of a motile variant derived from it by mutation produces trails as well as swarms. Perhaps some phage-borne genetic particles are of such a kind that they cannot be incorporated into the genetic organelle; or perhaps incorporation or its failure is only decided after the particle enters the recipient. If the latter is the case, it seems that the genetic material, if not at once incorporated, soon becomes incapable of incorporation. This might result either from some decay of the particle or from its becoming lodged in some special situation. Stocker (1953) suggested that transformation of pneumococci and transduction in *Salmonella* both resulted from the introduction into the organism of genetically active deoxyribonucleic acid (DNA), its side-by-side pairing with homologous material in a linear genetic organelle, and its consequent incorporation in continuity in a replica organelle in process of formation alongside the original one. On this model a failure of incorporation of part of an introduced fragment might result if one end of it became apposed to its homologue, and in consequence incorporated into a replica 'chromosome', while the other end, through failure to become apposed in time, remained as a side-arm when formation of the replica organelle was completed. If replication (or separation of replica from original) affected only genetic material arranged along the main length of the genetic organelle, then the side-arm would not be replicated, and would consequently be transmitted unilinearly. If a gene for formation of MC particles was carried on such a side-arm, abortive transduction of motility would be accounted for.

#### *Unilinear transmission of characters*

Investigation of abortive transduction of motility in *Salmonella* has revealed two instances of unilinear transmission of characters. When a unicellular (acellular) organism (or cell of a multicellular one) divides, the substance of the parent cell is distributed, equally or unequally, amongst the daughters; it

would therefore appear that all stable particulate cell structures and substances must be transmitted down one line of descent. There are, however, few reports of unilinear transmission of characters. This is presumably because the unilinear transmission of a particle will not produce striking effects if (i) the descendants not receiving the original particle synthesize new particles indistinguishable from it, or (ii) if the presence of a single particle (e.g. a protein molecule) in a cell is unrecognizable. In *Paramecium* the parental contractile vacuoles are transmitted to the daughters; but since each daughter also synthesizes new vacuoles indistinguishable from the old ones, the unilinear transmission of the parental vacuole is not observable. In bacteria there is evidence that molecules of an adaptive (inducible) enzyme are transmitted to the progeny even when synthesis has ceased owing to withdrawal of the inducer; each molecule of enzyme is presumably transmitted unilinearly, but this cannot be tested since the presence of a single enzyme molecule in a bacterium cannot be recognized.

Several instances of unilinear transmission have, however, been described. In a pedigree recorded by Jennings (1908) division of a misshapen *Paramecium* gave rise to a daughter with an abnormal protrusion or 'spine', whose unilinear transmission was observed for twenty generations. Jacob (1954) and Bertani (1954) found that the genes of a phage particle absorbed by a bacterium already lysogenic for a related phage persisted, but did not multiply, during several generations of bacterial growth. Lederberg (1956) described the unilinear transmission of formazan granules in *Salmonella*.

In many unicellular (or acellular) organisms (e.g. the yeasts) division is unequal, in that one daughter retains the parental cell wall; as might be expected, unilinear transmission of morphological characters has been observed in such organisms (Jennings, 1929; other instances are cited by Lederberg, 1956).

I wish to thank Dr C. Quadling and Mr H. A. Milne for preparing and photographing material used for illustrations.

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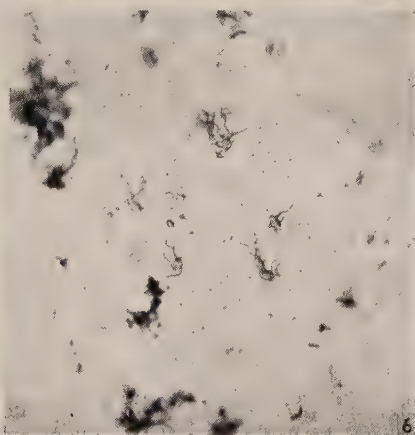
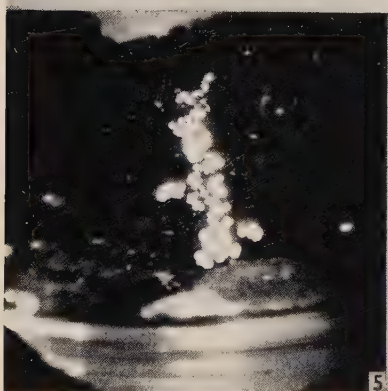
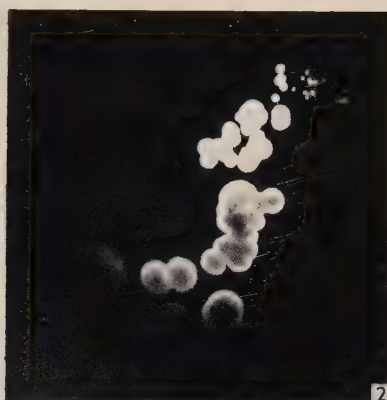
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## EXPLANATION OF PLATE

- Fig. 1. Trails produced by *Salmonella paratyphi-B* strain SW 966, treated with lysate of *S. typhimurium* strain TM 2; the treated organisms were plated at the centre of a 5 cm. Petri dish of semisolid medium, which was incubated at 37° for 42 hr.  $\times 1$ .
- Fig. 2. Distal (growing) end of one of the trails shown in Fig. 1.  $\times 8$ .
- Fig. 3. Droplet containing hundreds of non-motile and one motile organism, which has produced a streak (indicated by arrow) during 10 sec. exposure; dark-ground illumination.  $\times c. 300$ .
- Fig. 4. As fig. 3, but droplet contains one motile and thousands of non-motile organisms (smaller than those in fig. 3 because not in log phase).  $\times c. 300$ . (In figs. 3 and 4 the non-motile bacteria appear blurred, owing to Brownian movement.)
- Fig. 5. Trail produced by a motile 'initial' isolated from a lysate-treated culture of strain SW 541 and incorporated in a pour-plate of semisolid medium, which was incubated at 37° for 18 hr.  $\times c. 10$ .
- Fig. 6. Organisms of a motile variant obtained from *Salmonella typhimurium* strain SW 541 by transduction; flagella stained by the method of Leifson (1951).  $\times c. 650$ .

(Received 28 May 1956)





B. A. D. STOCKER—ABORTIVE TRANSDUCTION OF MOTILITY. PLATE 1

(Facing p. 598)



## Complementary Genes which Affect Penicillin Yields

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**SUMMARY:** Two loci which affect the production of penicillin by *Penicillium chrysogenum* have been identified. Capacity to produce penicillin is suppressed by eight mutant alleles at one of the loci, and a marked decrease of yield accompanied by an effect on conidium colour is produced by one mutant allele at the other locus. The two loci are on different chromosomes. The wild type alleles of the two genes restore penicillin production in the heterokaryotic and heterozygous conditions but not when they are carried in different mycelia in mixed culture. Haploid and diploid strains have been identified among the segregants from a heterozygous diploid.

In a previous work (Caglioti & Sermonti, 1956) five strains of *Penicillium chrysogenum* Thom which had been rendered non-producers of penicillin by four independent mutations were studied. Balanced heterokaryons and heterozygous diploids were synthesized, combining two by two four mutants in all possible ways, but no production of penicillin was obtained from any combination. Thus it was shown that the five genotypes in question are not complementary in respect of penicillin biosynthesis. The present work was planned with a view to extending these studies. One of the strains used in the previous work was taken as a test strain, and five other non-producing mutants were tested for their complementarity with it. A positive result has been obtained in this second attempt.

### METHODS

The media, techniques and symbols used in the present paper are the same as those used in the previous paper (Caglioti & Sermonti, 1956) to which the reader is referred. Shake-flask submerged cultures were standardized as follows.

*Submerged culture.* Spores for the seed flask were collected from week-old cultures on husked barley moistened with a sporulating solution consisting of 3% (w/v) glycerol and 0.1% (w/v) asparagine in water. A dense suspension of spores was inoculated in 500 ml. Ignis glass flasks having 40 mm. mouths and cotton-wool plugs, each containing 100 ml. of the following medium: 80 g. corn steep liquor; 60 g. dextrin; tap water to 1 l. The pH value was brought to 5.2 with NaOH before autoclaving at 120° for 20 min. After 48 hr. of incubation 5 ml. of the culture were transferred to flasks of the same type containing 60 ml. of the following medium: 70 g. corn steep liquor; 4.5 g. lactose; tap water to 1 l. The pH value was 4.6 before sterilization (as above); no correction of pH value was made. CaCO<sub>3</sub> (0.6 g.; separately sterilized) was then added to each flask. Other additions, per flask, were: 6% (w/v) anti-foam

E 100 (made by Bayer-Leverkusen) in lard oil, 0.1 ml. added with inoculum; 10 % (w/v) sodium phenylacetate, 0.3 ml. every 24 hr. starting at 24 hr. The cultures were incubated at 24° on a rotary shaker (Paladino, 1954).

*Organisms.* Strain 65 *pr*  $y_3 p_2$ , a non-penicillin-producing strain of *Penicillium chrysogenum* (Caglioti & Sermonti, 1956), was used as test strain and was crossed with the new strains to be tested. The origin and characteristics of the mutants obtained are set out in Table 1. Strains 93  $p_5$ , 98  $p_6$ , 109  $p_7 y_1$  and 110  $p_8 y_1$  kept their morphological characteristics after losing capacity to produce penicillin by mutation. Strain 96  $p_9$  showed a diminution of its production capacity to about a tenth that of the original strain, accompanied by a grey coloration of the conidia. The hypothesis that these two modifications are the result of a single mutation is suggested by their simultaneous appearance and by the fact that the two characters in question always segregated together from heterozygous diploid XXIX (see below).

Table 1. *Mutants of Penicillium chrysogenum obtained in the course of the present work*

Strain symbol*	Parent strain	Conidium colour	Nutritional requirement	Comparative penicillin yield† (47.1564 Wis. = 100)
47.1564 Wis.	—	Green	None	100
15 $y_1$	47.1564 Wis.	Yellow	None	113.9
96 $p_9$	47.1564 Wis.	Grey	None	10.2
98 $p_6$	47.1564 Wis.	Green	None	0
93 $p_5 y_1$	15 $y_1$	Yellow	None	0
109 $p_7 y_1$	15 $y_1$	Yellow	None	0
110 $p_8 y_1$	15 $y_1$	Yellow	None	0
147 <i>ad</i> $p_9$	96 $p_9$	Grey	Adenine	—
162 <i>nic</i> $p_9$	96 $p_9$	Grey	Nicotinamide	—
163 <i>ly</i> $p_9$	96 $p_9$	Grey	Lysine	—
158 <i>hy</i> $p_6$	98 $p_6$	Green	Hypoxanthine	—
144 <i>me</i> $p_5 y_1$	93 $p_5 y_1$	Yellow	Methionine	—
148 <i>me</i> $p_7 y_1$	109 $p_7 y_1$	Yellow	Methionine	—
157 <i>hy</i> $p_8 y_1$	110 $p_8 y_1$	Yellow	Hypoxanthine	—

\* The strain symbols consist of a code number followed by symbols as follows: *y* = yellow conidium colour; *p* = absence or marked reduction in penicillin production; *ly* = lysine requirement; *ad* = adenine requirement; *nic* = nicotinamide requirement; *hy* = hypoxanthine requirement; *me* = methionine requirement. Different subscripts indicate different mutations.

† Penicillin yields at day of maximum titre in submerged culture. Three flasks in each run. Maximum yield of 47.1564 Wis: 620 units/ml. at 120 hr.

## RESULTS

### *Investigation of mutants $p_5$ , $p_6$ , $p_7$ and $p_8$*

The first part of the present work concerned the study of strains 93  $p_5 y_1$ , 98  $p_6$ , 109  $p_7 y_1$  and 110  $p_8 y_1$ . Four suitably marked derivatives from these strains (144 *me*  $p_5 y_1$ , 158 *hy*  $p_6$ , 148 *me*  $p_7 y_1$  and 157 *hy*  $p_8 y_1$ , respectively) were used in the synthesis of four balanced heterokaryons with the strain 65 *pr*  $y_3 p_2$ . All the heterokaryons were obtained as syntrophic tufts on a limiting medium made up of a 1 : 5 mixture of complete and minimal medium (Sermonti &



Spada-Sermonti, 1954). From these four heterokaryons were isolated the corresponding four heterozygous diploids, XXII (65/144), XXIII (65/157) and XXIV (65/158) as sectors from heterokaryons, and diploid XXVII (65/148) as a colony growing on minimal medium after plating spores of the corresponding heterokaryon.

The diploids were purified by isolation of single conidia with a micro-manipulator (Sermonti, 1954*a*). They were all prototrophic and had green conidia as was to be expected, since the factor  $y_3$  of strain 65 was already known to be non-allelic to  $y_1$  of strains 93, 109 and 110, and both are known to be recessive (Caglioti & Sermonti, 1956). In submerged culture all the heterozygous diploids proved to be non-penicillin-producing, like their component strains (see Table 2). This part of the work thus confirms and extends the results of Caglioti & Sermonti's (1956) paper.

#### Investigation of mutant $p_9$

The second part of the work concerns the study of strain 96  $p_9$ . Three different mutants (147 *ad*  $p_9$ , 162 *nic*  $p_9$  and 163 *ly*  $p_9$ ) were derived from this strain and used to synthesize three balanced heterokaryons with the test strain 65 *pr*  $y_3 p_2$  by the techniques already mentioned. The three corresponding heterozygous diploids, XXXII (65/147), XXXIII (65/162) and XXIX (65/163) were isolated as sectors from the balanced heterokaryons. They were purified by isolation of a single conidium and proved to be prototrophic with green spores. On testing in submerged culture they turned out to be normal penicillin producers (see Table 2).

Table 2. *Penicillin production in submerged culture by heterozygous diploids between strains mutant for penicillin-producing capacity*

Code	Heterozygous diploids Component strains	Penicillin production* (units/ml.)	
		96 hr.	120 hr.
XXII	65 <i>pr</i> $y_3 p_2$ /144 <i>me</i> $p_5 y_1$	0.2	0.3
XXIII	65 <i>pr</i> $y_3 p_2$ /157 <i>hy</i> $p_8 y_1$	0.2	0.3
XXIV	65 <i>pr</i> $y_3 p_2$ /158 <i>hy</i> $p_8$	0	—
XXVII	65 <i>pr</i> $y_3 p_2$ /148 <i>me</i> $p_7 y_1$	0	—
XXIX	65 <i>pr</i> $y_3 p_2$ /163 <i>ly</i> $p_9$	342	436
XXXII	65 <i>pr</i> $y_3 p_2$ /147 <i>ad</i> $p_9$	215	244
XXXIII	65 <i>pr</i> $y_3 p_2$ /162 <i>nic</i> $p_9$	291	307
XXXV	63 <i>cy</i> $p_3 y_1$ /163 <i>ly</i> $p_9$	175	212
47.1564 Wis. (haploid)		350	551

\* Every figure is the average of two values.

Fig. 1 shows the rate of penicillin production by diploid XXIX 65 *pr*  $y_3 p_2$ /163 *ly*  $p_9$  in shake flasks as compared with those of strains 99  $p_2$  and 96  $p_9$  and with the wild type strain 47.1564 Wis. from which both are descended. In the same figure is shown the rate of penicillin production by a mixed culture of strains 99  $p_2$  and 96  $p_9$  (see below). Production by the diploid strain is

practically as good as that of the wild type strain, while the component strains give very reduced yields ( $96 p_9$ ) or none at all ( $99 p_2$ ).

A heterozygous diploid (XXXV) was synthesized between the non-penicillin-producing strain  $63 cy p_3 y_1$  (Caglioti & Sermonti, 1956) and strain  $163 ly p_9$  by using the same techniques. On testing in submerged culture this strain also proved to be penicillin-producing, but its yield of penicillin was less than those

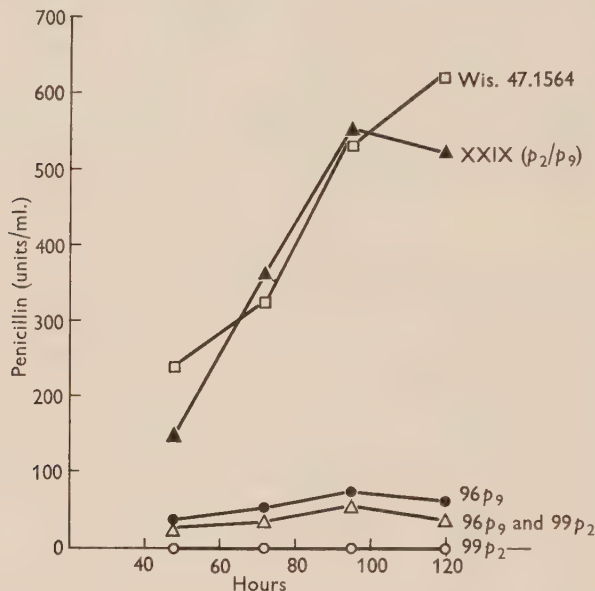


Fig. 1. Penicillin production in submerged culture by strains: 47.1564 Wis.;  $99 p_2$ ;  $96 p_9$ ;  $99 p_2$  and  $96 p_9$  in mixed culture; diploid XXIX  $65 pr y_3 p_2/163 ly p_9$ . Medium: corn steep, lactose. Four flasks in each run.

of heterozygous diploids containing the allele  $p_2$  (Table 2). The marked phenotypic expression of allele  $p_3$  in the heterozygous state has already been remarked upon (Caglioti & Sermonti, 1956).

#### *Production of penicillin by $p_9 + p_2$ heterokaryons and $p_9$ and $p_2$ mixed culture*

With a view to determining the level at which the complementary effect between the genotypes  $p_2 P_9$  and  $P_2 p_9$  takes effect, two balanced heterokaryons carrying these allele combinations and a mixed culture of the strains  $99 p_2 P_9$  and  $96 P_2 p_9$  (referred to above as  $99 p_2$  and  $96 p_9$ ) were examined for their penicillin production. The balanced heterokaryons were tested in surface culture on minimal agar (Jarvis & Johnson, 1950) with the technique described by Caglioti & Sermonti (1956). Auxanographic tests were used (Sermonti, 1954a) to check the heterokaryotic nature of these colonies. Table 3 gives the data for penicillin production; it appears from this table that the penicillin yield of balanced heterokaryons is perfectly comparable with that of heterozygous diploid XXIX and the wild type strain 47.1564 Wis.

The mixed culture of strains  $96 P_2 p_9$  and  $99 p_2 P_9$  was effected in shake flasks. The two strains were separately seeded in the seed flasks, and after 48 hr. 2.5 ml. of each culture were transferred into one flask of the same fermentation medium. The experiment was conducted simultaneously with that described in the last section and reported in Fig. 1; the results are also shown in Fig. 1. The penicillin yield of the mixed culture was very low, roughly intermediate between those of the separate component strains.

Table 3. *Penicillin production in surface culture on minimal agar by two  $p_2 + p_9$  heterokaryons compared with a  $p_2/p_9$  heterozygous diploid and other strains*

A plug of agar is removed from the edge of the colony and placed on agar inoculated with *Bacillus subtilis*. After incubation overnight at 37° the inhibition halo is measured. Penicillin production is calculated by reference to a standard curve (yield of diploid XXIX at the 11th day = 500). Each figure is the average of four values, two from each of two distinct cultures. Different cultures were sampled on different days.

Age of the culture in days	Strains					
	$96 p_9$	$99 p_2$	XXIX 65/163 ( $p_2/p_9$ )	65 + 163 ( $p_2 + p_9$ )	65 + 162 ( $p_2 + p_9$ )	47.1564 Wis.
6	5	0	37	32	37	55
7	35	0	170	150	170	220
11	129	0	500	—	530	550

#### *Variation of pH value in the culture medium*

Penicillin production is particularly sensitive to changes in pH value of the medium. Good production may be obtained at pH values between 6.8 and 7.8 (Johnson, 1953). The pH value of the culture media was constantly checked during the runs. In cultures of strain 47.1564 Wis. the pH value 24 hr. after inoculation was generally 7; it then fell to values between 6.5 and 6.8 at 48 hr., to rise again until at 120 hr. it reached about pH 8. In cultures of strain  $96 p_9$  the pH decrease at 48 hr. was appreciably more marked, but the pH value then returned to optimal values. In cultures of strain  $99 p_2$  the flexion at 48 hr. was not observed, and the pH value kept to optimal values for a longer period. Thus for a large part of the run with either strain the pH of the medium was perfectly compatible with normal penicillin production. The behaviour of the pH value in runs with heterozygous diploid XXIX ( $p_9/p_2$ ) was intermediate between those of strains  $96 p_9$  and  $99 p_2$ , and that of the mixed culture  $96 p_9$  and  $99 p_2$  was very similar to that observed in the diploid culture. Yet the diploid gave normal penicillin yields, while yields from the mixed culture were very low.

#### *Genetic analysis of diploid XXIX*

*Size of conidia of segregant strains.* Diploid XXIX  $65 pr y_3 p_2/163 ly p_9$  was genetically analysed by an examination of its somatic segregation. Attention was paid to the size of the conidia of the segregant strains, which has not previously been done in analyses of segregants from diploids of *Penicillium*

*chrysogenum* (Sermonti, 1954*b*; Caglioti & Sermonti, 1956; Sermonti, to be published). It was possible to classify the various clones into two groups according to the size of their conidia: some presented conidia of an average diameter close to that of the original diploid, others close to that of the haploid component strains (Table 4). The measurements were carried out on conidia

Table 4. *Average volume and diameter of the conidia of diploid XXIX, its component strains and its segregants*

Strain		Size of conidia		Classification
Code	Phenotype*	Diameter† ( $\mu$ .)	Corresponding volume ( $\mu^3$ )	
65	<i>pr y<sub>3</sub> p<sub>2</sub></i>	3.98	33.0	Haploid
163	<i>ly p<sub>0</sub></i>	3.83	29.4	Haploid
XXIX	+	5.24	75.3	Diploid
Segregants				
697.1	<i>pr</i>	4.10	36.1	Haploid
697.4	<i>pr</i>	4.18	38.2	Haploid
714.133	<i>pr</i>	3.97	32.8	Haploid
714.144	<i>pr</i>	3.84	29.6	Haploid
697.6	<i>ly p</i>	3.73	27.2	Haploid
642.1	<i>ly p pr</i>	4.98	64.7	Diploid
714.138	<i>pr</i>	4.98	64.7	Diploid
714.118	<i>pr</i>	5.24	75.3	Diploid
642.3	<i>y p</i>	5.04	67.0	Diploid
714.4	<i>y p</i>	5.21	74.0	Diploid
697.12	<i>pr</i>	5.15	71.4	Diploid
745.14‡	<i>pr ly p</i>	5.36	80.6	Diploid

\* The allele symbols indicate the phenotype in both haploids and diploids.

† The diameter given is the average taken from measurements of 50 conidia in each case.

‡ See Table 5.

collected with a loop in a drop of water containing *c.* 10 % sodium lauryl-sulphonate; this was placed on a microscope slide and covered with a cover-glass. The conidia were isolated or, exceptionally, were in very short chains, and of various shapes ranging from spherical (the commonest) to ellipsoidal. Measurements were made of the diameter of the spherical conidia, and of the major and minor axes of the ellipsoidal conidia, taking the average between them. The measurements were carried out with a Spencer eyepiece micrometer (calibrated: 1 division = 1.70  $\mu$ ., useful approximation 1/10 of a division). The averages given in Table 4 are from measurements of 50 conidia for each strain. The overall average diameter of the conidia of strains with normal conidia was 3.95  $\mu$ . and of strains with giant conidia 5.15  $\mu$ .; the ratio between these two values is 1.3, corresponding to a ratio between volumes of about 2.

In the case of the strains not included in Table 4 enough conidia were measured to classify the strain with reasonable certainty in one of the two groups. Some colonies presented conidia of extremely variable size: these were purified by isolation of single conidia and the diameter of conidia measured in the pure colonies.

The segregants selected included a very high proportion of clones with



normal conidia; giant conidia were found in only eight segregants out of a total of 106 (see Table 6).

Four segregants bearing normal conidia and four segregants bearing large conidia were purified by isolation of single conidia with a micromanipulator and examined for further segregation; the latter produced further segregants, showing that they still possessed heterozygous genes, while the former produced no second-order segregants (Table 5). These results suggest, especially in view of the findings of Pontecorvo, Tarr Gloor & Forbes (1954) on mitotic segregation in *Aspergillus nidulans*, that the segregants bearing small conidia are haploid and the segregants bearing large conidia diploid. They will be so considered here.

Table 5. *Second-order segregation from some first-order segregants from diploid XXXI related to conidia volume*

First-order segregants		Conidium diameter* ( $\mu$ .)	Colonies examined		Segregants obtained	
			Selection for	Total examined (no.)	(no.)	(phenotype)
Code	Phenotype†					
A. Segregants with giant conidia						
714.138	<i>pr</i>	4.98	Colour	660	1	<i>y pr</i>
714.118	<i>pr</i>	5.24	Colour	324	2	<i>y ly pr p</i> ‡ <i>ly pr p</i> §
714.4	<i>y p</i>	5.21	Require- ments	2264	4	<i>pr y p</i>
679.12	<i>pr</i>	5.15	Colour	1700	1	<i>p pr</i>
B. Segregants with normal conidia						
714.144	<i>pr</i>	3.84	Colour	2000	0	—
714.133	<i>pr</i>	3.97	Colour	1300	0	—
697.4	<i>pr</i>	4.18	Colour	1694	0	—
697.1	<i>pr</i>	4.10	Colour	1368	0	—

\* See Table 4.

† The allele symbols indicate the phenotype in both haploids and diploids.

‡ Very poor growth on MM + proline + lysine and none on MM + proline.

§ Strain 745.14, see Table 4.

*Phenotypes of somatic segregants from diploid XXIX.* Colour segregants were obtained by plating conidia of the diploid on complete medium and picking out the colonies of a colour different from the rest of the population after 1 week of growth. Yellow and grey (or at least paler than normal) colonies were transferred to fresh complete agar. Many of the pale colonies in time assumed a green colour similar to that of the wild type, darker than that of the diploid colonies. These all turned out on later testing to be haploid and to have the proline requirement. Thirty-six other colonies of a yellowish green colour were tested for requirements and conidia size; all proved to be diploid, and there were thirty-four prototrophic and two with proline requirement. Deficient segregants were selected as poorly growing colonies after plating conidia of diploid XXIX on limiting medium (Sermonti, 1954*b*). All the segregants were checked for colour, tested for nutrient deficiencies and

penicillin production on agar (Caglioti & Sermonti, 1956), and average conidia size measured. The phenotypes of the segregants selected by the two techniques are reported in Table 6.

Table 6. *Segregants\* selected from diploid XXIX 65 pr y<sub>3</sub> p<sub>2</sub>/163 ly p<sub>9</sub>*

	Selected for colour†	Selected for requirements‡
A. Haploid		
Yellow: <i>pr p<sub>2</sub></i>	1	1
Grey: <i>ly p<sub>9</sub></i>	33	5
Green§: <i>pr P</i>	53	5
	87	11
B. Diploid		
Yellow: <i>pr p<sub>2</sub></i>	1	0
<i>p<sub>2</sub></i>	3	0
Green: <i>pr P</i>	2	2
	6	2

\* The allele symbols indicate the phenotype in both haploids and diploids. *P* = produces penicillin.

† Out of 3698 colonies observed in one experiment. One yellow *pr p<sub>2</sub>* diploid and one yellow *p<sub>2</sub>* diploid from 912 colonies observed in another experiment.

‡ 75 colonies tested out of 750 observed on limiting medium.

§ The green colour of these strains changes from greyish to the dark green of the wild type, while the colour of the diploids is yellowish green.

All the mutant alleles of the component strains of diploid XXIX segregate from this strain, including *p<sub>2</sub>*, complete incapacity to produce penicillin, and *p<sub>9</sub>*, great diminution in penicillin yield accompanied by grey colour of the conidia; *p<sub>2</sub>* is linked to *y<sub>3</sub>* but not to *pr*, and *p<sub>9</sub>* is linked to *ly*.

Table 7 shows the penicillin yield in submerged culture of some of the

Table 7. *Penicillin production in submerged culture by diploid XXIX, its component strains and its segregants*

Code	Phenotype*	Ploidy†	Penicillin production‡ (units/ml.)	
			76 hr.	96 hr.
96	<i>p<sub>9</sub></i>	Haploid	62	69
163	<i>ly p<sub>9</sub></i>	Haploid	41	51
99	<i>p<sub>2</sub></i>	Haploid	0	0
65	<i>pr y<sub>3</sub> p<sub>2</sub></i>	Haploid	1.0	2.2
XXIX	+	Diploid	374	475
Segregants				
642.3	<i>y p</i>	Diploid	0.6	1.9
697.16	<i>pr y p</i>	Haploid	0.6	1.2
697.6	<i>ly p</i>	Haploid	12	35
697.7	<i>ly p</i>	Haploid	19	38
697.4	<i>pr</i>	Haploid	481	728
697.8	<i>pr</i>	Haploid	437	708

\* The allele symbols indicate the phenotype.

† The ploidy is determined on the basis of conidial measurements.

‡ Every figure is the average of two values.

segregant strains. Three degrees of penicillin production may be observed: haploid segregants for the proline requirement alone yield more than 700 units penicillin/ml., i.e. the highest yields given by 47.1564 Wis. and higher than those of the original diploid; segregants for the lysine requirement give less than a tenth of the diploid yield; yellow segregants give only traces of penicillin. Two diploid proline-dependent segregants were compared for penicillin production on agar with five haploid proline-dependent segregants, and proved to produce nearly half the yield of the latter.

#### DISCUSSION

The present work has led to the identification of a second locus which controls penicillin production in addition to the one identified by Caglioti & Sermoni (1956). The nine mutant alleles which have now been examined (five in the present work and four in the previous one) include eight which belong to the same locus and one ( $p_9$ ) belonging to a second locus. The last differs from the others in exerting an effect on conidial colour and in its incomplete suppression of penicillin-producing capacity in the haploid.

The isolation of haploid segregants from the heterozygous diploid XXIX is the first instance of the complete cycle of parasexual recombination (haploid-diploid-haploid) to be reported in *Penicillium chrysogenum*. The presence of haploid as well as diploid segregants is a further parallel in somatic segregation behaviour between *Aspergillus nidulans* (Pontecorvo *et al.* 1954) and *P. chrysogenum*. The haploid segregants in *Aspergillus* are thought to be the result of irregular distribution of whole chromosomes at mitosis (Pontecorvo *et al.* 1954). If a similar process be assumed for the formation of haploid nuclei in *P. chrysogenum*, the two genes  $p_2/P_2$  and  $p_9/P_9$  must be on different chromosomes, since the haploid segregants of diploid XXIX include a number giving a penicillin production as high as that of the starting strain (47.1564 Wis.). If the two genes were on the same chromosome, one mutant allele or the other would necessarily be present in each haploid segregant. In any case, the qualitative data of the segregation from diploid XXIX, and still more the submerged culture fermentation results with some of the segregants, indicate the genetic nature of the mutations  $p_2$  and  $p_9$ .

The following conclusions may be drawn with respect to the genotypes  $p_2 P_9$  and  $P_2 p_9$ . Neither determines normal penicillin production in the haploid. They do not permit normal penicillin production when they are both present in the same culture medium but in different mycelia. Penicillin production is restored when they are carried in the same cytoplasm in a balanced heterokaryon, and also when they are together in heterozygous diploid nuclei. A further appreciable increase in production occurs when the two alleles  $P_9$  and  $P_2$  are together in the same haploid nucleus in the absence of the corresponding recessive alleles.

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## Gene Recombination in *Streptomyces coelicolor*

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**SUMMARY:** Prototrophic recombinant strains were obtained by plating on minimal medium spores from mixed cultures grown on limiting agar, of two strains each with a different double nutritional requirement. Clones showing new associations of properties were isolated on partially supplemented media. Some showed one 'mutant' character derived from one of the starting strains and 'wild' characters derived from the other; others showed two 'mutant' characters, each derived from one of the starting strains.

Actinomycetes are generally regarded as asexual micro-organisms. However, different parasexual processes have been discovered in the last ten years in a few species of bacteria and asexual fungi (Lederberg & Tatum, 1946; Zinder & Lederberg, 1952; Pontecorvo, Roper & Forbes, 1953; Pontecorvo & Sermonti, 1954) leading to gene recombination otherwise than by sexual reproduction. The properties of the actinomycetes are intermediate between those of the bacteria and the fungi, so that the existence of a process of parasexual recombination was to be expected in this group of micro-organisms as well. A preliminary report of the present work appeared elsewhere (Sermonti & Spada-Sermonti, 1955).

### METHODS

*Organisms.* A strain of *Streptomyces coelicolor* Reiner-Müller, supplied by the courtesy of Dr A. Tonolo of this laboratory, was used. The colonies consist of a colourless primary mycelium and a thick layer of whitish secondary or aerial mycelium. The medium becomes intensely coloured by a blue pigment formed by the organism (Tonolo, Casinovi & Marini-Bettolo, 1954). The authors were also supplied by Dr Tonolo with an achromogenic variant in which the primary mycelium is red and the aerial mycelium greyish pink.

*Media.* The media employed were as in the genetic research on *Penicillium chrysogenum* carried out in this laboratory (Sermonti, 1954): viz. a minimal medium (MM) (modified Czapek-Dox) and a complete medium (CM) containing yeast extract, casein hydrolysate, nucleic acid hydrolysate, corn steep and a mixture of vitamins. All the cultures were incubated at 30°.

*Isolation of mutants.* The spores were subjected to ultraviolet irradiation after surface plating on CM agar (Pontecorvo, 1953) to give a mortality ranging from 98 to 99.7 %. Mutants were selected from the surviving colonies by the total isolation method (Fries, 1948). At first, spores from every surviving colony were point-transferred to MM agar; later, spores collected from each single colony were streaked on to MM agar by means of a loop containing a drop

of sterile water. The latter procedure turned out to be more efficient and was adopted later for recognition of nutritional requirements of recombinant strains. The clones which showed no growth on MM were recovered and subjected to Beijerinck's auxanographic test. A list is given in Table 1 of 19 mutants obtained by this procedure. Nine different types of mutant with determined nutritional requirements were isolated, and two mutants whose requirements were not determined. One of these latter (*4 un pigm*) grows on MM supplemented with casein hydrolysate, and the other (*18 un nic pigm*) requires added peptones. In four of the experiments the rate of mutant occurrence was higher than 1 % of the colonies tested.

Table 1. *Isolation of biochemical mutants in Streptomyces coelicolor*

Ultraviolet irradiation of Petri dishes of CM agar each plated with about 10,000 spores. Survival rate between 2 and 0.3 %. Total isolation of surviving colonies.

Strain irradiated. Symbol*	Colonies isolated (no.)	Mutants among isolates	
		(no.)	Symbol*
1+	725	1	3 <i>hist</i>
2 <i>pigm</i>	1565	1	4 <i>un pigm</i>
3 <i>hist</i>	2112	1	5 <i>me hist</i>
2 <i>pigm</i>	1179	4	6 <i>glu pigm</i>
			7 <i>as pigm</i>
			8 <i>nic pigm</i>
			9 <i>nic pigm</i>
7 <i>as pigm</i>	336	4	10 <i>cys as pigm</i>
			11 <i>me as pigm</i>
			12 <i>me as pigm</i>
			13 <i>arg as pigm</i>
6 <i>glu pigm</i>	346	2	14 <i>pr glu pigm</i>
			15 <i>arg glu pigm</i>
3 <i>hist</i>	180	2	16 <i>me hist</i>
			17 <i>ur hist</i>
8 <i>nic pigm</i>	566	1	18 <i>un nic pigm</i>

\* The symbols consist of a code number and the symbols of the induced mutations in inverse order of induction with the following meanings: *arg*=arginine requirement; *as*=aspartic acid requirement; *cys*=cysteine requirement; *glu*=glutamic acid requirement; *hist*=histidine requirement; *me*=methionine requirement; *nic*=nicotinamide requirement; *pigm*=absence of blue pigment; *pr*=proline requirement; *un*=unknown requirement; *ur*=uracil requirement; + represents the wild type.

## RESULTS

### *Production of 'syntrophic tufts'*

The first attempts to set up a parasexual process were along the lines followed successfully in the experiments with *Penicillium chrysogenum* (Sermonti, 1954): isolation of tufts of exuberant growth between colonies with complementary requirements growing on limiting medium. The first strains to be employed were 3 *hist* and 4 *un pigm*. A suspension, containing about 2000 spores/ml., of each of the two strains was spread at the rate of 0.1 ml. (200 + 200 spores) per plate on the surface of a series of Petri plates containing

MM mixtures of MM and CM in various proportions (MM : CM = 1 : 20, 1 : 4 and 1 : 1) or CM agar. After 4 days of incubation tufts of exuberant growth ('syntrophic tufts') appeared in the areas of contact between colonies of the two types on all the dishes except those containing MM alone. These resembled those already observed in *P. chrysogenum* (Sermoniti, 1954). They were very obvious on the seventh day, and in sharp contrast with the adjacent colonies in height and richness of spore formation (see Pl. 1, figs. 1, 2). Only a very slight growth, probably due to strain *4 un pigm*, was observed on MM. The tufts were especially noticeable on the 1 : 1 MM : CM media and on CM. The strains used showed slightly less growth on this latter medium, with spores forming either very late or not at all. Increase of the quantity of MM in the medium led to greatly decreased growth. Transfer of tufts to MM gave rise in some cases to very stunted colonies with an irregular edge, very delayed spore formation limited to the centre of the colony, and late and scanty production of the blue pigment. No growth was observed after plating on MM several tens of thousands of spores collected from one of these colonies; on CM, colonies of the two component types reappeared, forming exuberant tufts in their contact zones. The same results were obtained on plating several tens of thousands of hyphal fragments in a suspension obtained by grinding finely in distilled water the spore-free margin of a colony. The experiments were discontinued, since strain *4 un pigm* showed traces of growth on plating on MM.

Syntrophic tuft production was next tried with strains *5 me hist* and *14 pr glu pigm* on CM, following the same procedures. In this case the occurrence of the phenomenon was sporadic and concentrated in particular areas of the dish. Sixteen tufts were transferred to MM agar, and produced irregular colonies like those already described. Two of these after a few days produced sectors of greater height and richer spore formation, which overgrew the original colony (Pl. 2, fig. 3). A spore suspension (about 1000 spores/ml.) from one of these sectors was well broken up so that it consisted almost entirely of single spores and was then plated on CM and on MM agar at the rate of 0.1 ml./dish. Thirty colonies grew on three CM agar dishes, as against 267 colonies on four MM agar dishes. All the colonies produced the blue pigment. Ninety-six colonies were transferred from the CM agar to fresh MM agar, and turned out to be prototrophic and to produce the blue pigment. One of the colonies which had been grown on CM agar was used to obtain a spore suspension, which was again plated on fresh CM agar. A total of 17,700 colonies was observed on fifty Petri plates, and all of them turned out to be blue pigment-producing. Sixty-two of the more stunted colonies were transferred to MM agar and turned out to be prototrophic and pigment-producing.

Several preliminary experiments were carried out to determine conditions for regular reproducibility of the formation of syntrophic tufts. Strains *3 hist* and *8 nic pigm* did not produce syntrophic tufts on CM, but the phenomenon appeared regularly with these strains on CM enriched with yeast autolysate at a concentration of 1-2 % (w/v). The two strains produced no spores on this medium. The colonies formed were prominent and gelatinous, with tufts looking like opaque white spindles.



*Mixed cultures on agar slopes of limiting medium*

A search was made for a procedure to obtain syntrophic growth, like that observed in the zone of contact between two colonies in the experiments just described, but homogeneously over the whole of an agar surface. This was achieved by spreading a thick mixed suspension of spores of two different kinds on the surface of agar slopes of limiting agar. The limiting agar was obtained as already described, by mixing CM and MM in various proportions. Two strains, each with two requirements, were used: *5 me hist* and *14 pr glu pigm*. Separately streaked on CM agar slopes, these strains grew well and formed spores. They also grew well and formed spores freely when grown on MM supplemented by the substances on which they were dependent, in the following concentrations: 0.68 mM-DL-methionine, 1.30 mM-L-histidine HCl, 1.30 mM-L-proline, 11.8 mM-glutamic acid. Growth was poor and spores were not formed on limiting media composed of a mixture of MM and CM in the proportion 1 : 2.3, or with lower proportions of CM (see Pl. 2, fig. 4). Strain *5 me hist* produced no pigment under these conditions.

On streaking together spores of the two strains in approximately equal quantities (about 200,000 of each strain per tube) on agar slopes of limiting medium, exuberant and precocious growth was produced (Pl. 2, fig. 4). By the

Table 2. *Selection on minimal agar of prototrophic colonies derived from mixed culture of strains 5 me hist and 14 pr glu pigm, and controls*

Spores derived from	Spores plated		Prototrophic colonies	
	Total (no.)	Per plate (no.)	Total (no.)	Per plate (no.)
Strain <i>5 me hist</i>	$24.7 \times 10^6$	$1.30 \times 10^6$	0	0
Strain <i>14 pr glu pigm</i>	$12.0 \times 10^6$	$0.75 \times 10^6$	0	0
Separate { <i>5 me hist</i> cultures* { <i>14 pr glu pigm</i>	$33.6 \times 10^6$ †	$1.98 \times 10^6$	0	0
Mixed { <i>5 me hist</i> culture‡ { <i>14 pr glu pigm</i>	$46.8 \times 10^6$ †	$1.17 \times 10^6$	10,832	271

\* Spores obtained from separate cultures on CM and mixed together at the moment of plating on MM agar. 22 % strain *14*.

† Corrected for viable counts.

‡ Spores obtained from 4-day old mixed culture on MM+CM (1:1) agar slopes. 17 % strain *14*.

third day, spore formation was heavy and the blue pigment had been densely produced. No trace of growth was observed on MM agar slopes. A suspension of spores from a mixed (MM:CM = 1:1) 4-day culture, in distilled water, was filtered through a Permax gamma filter to eliminate fragments of mycelium, and washed by centrifugation in distilled water; it was then plated at the density of about  $1.4 \times 10^6$  spores/plate on forty MM agar plates. Part of the suspension was plated on four CM agar plates at 100 spores/plate; 342 colonies grew on CM, of which 61 (17 %) showed the red colour of strain *14* and the rest produced the blue pigment. Colonies began to appear on the MM agar on the third day



after plating. On the fifth day they were small but prominent and had formed spores (Pl. 2, fig. 5), and the blue pigment was beginning to diffuse into the medium. The average number of colonies/plate was calculated to be  $271 \pm 6$ , equal to 0.023 % of the viable spores plated. As a control, strains 5 and 14 separately and a mixed suspension of spores of the two strains obtained from separate culture, were plated on MM agar (Table 2). No growth on MM was observed after 2 weeks, either from the spores of the two separate strains or from the mixed suspension. The conclusion was drawn that the colonies which grew on MM from spores of the mixed culture were to be regarded as prototrophic recombinants.

Isolation of prototrophic recombinants was carried out on MM from another nine mixed culture agar slopes of strains 5 and 14. Eight tubes gave positive results, and the frequency of prototrophic recombinants from one of them was over 40 % of the viable spores plated. Prototrophic recombinants were also obtained from a mixed culture on limiting medium of strains 8 *nic pigm* and 5 *me hist* at a rate of 0.029 % of the viable spores plated.

#### *Segregants from prototrophic recombinants*

Attempts were made, on the hypothesis that the recombinants obtained were heterozygous diploids (Roper, 1952), to obtain from the prototrophic recombinants described in the last section strains in which one or more of the markers of the parent strains was present. Spores of some of the prototrophic recombinants were plated on CM agar and hundreds of the colonies formed were observed for colour or tested, but without success in any case, with the exception of one colony which was found to have numerous red sectors, and from which three non-pigment-producing clones were isolated.

The spores were subjected to ultraviolet irradiation in order possibly to increase the frequency of any segregation process; the surviving clones were tested for prototrophism once in the first generation and once in the second. No segregants were found. The attempts were discontinued at this stage and the study of auxotrophic recombinants as described in the next section begun. The experiments described above, however, indicate the stability of the prototrophic recombinants tested.

#### *Auxotrophic recombinants obtained directly from mixed cultures*

After this lack of success in obtaining segregants from prototrophic recombinants, a search was made for auxotrophic recombinants directly from the mixed cultures on limiting medium. Spores from mixed cultures of strains 5 and 14 were plated on MM agar supplemented with pairs of the nutrients required by the strains used, in all possible combinations except those which would permit the growth of either parental strain (Table 3); the nutrients were used in the concentrations already given. Colonies which developed on these media were transferred by streaking (see above) to MM agar. Some of them did not grow. The MM agar under each streak of these clones was divided into two parts (see above) and transferred to two minimal media, one supplemented with one of the nutrients present in the original partially supplemented

medium, and the other with the other. The clones which grew on one only of these media were regarded as dependent on the substance added to the medium on which they did grow. Two clones which grew on neither of the two media were transferred to CM and tested by the auxanographic technique. Both showed requirements for both methionine and proline. Many other strains with a single requirement were tested by the auxanographic technique.

Table 3. *Study of new recombinant types from the spores of a mixed culture of 5 me hist and 14 pr glu pigm*

Mixed culture medium (MM:CM)	Age of the mixed culture (days)	Medium on which spores plated (MM supplemented by)*	Rate of occurrence of recombinants (%)	Colonies tested†	Recombinant types obtained	
					(no.)	(type)‡
1:2:3	7	Proline and histidine	3.1	36	3	<i>pr</i>
					4	<i>hist</i>
					29	+
1:2:3	7	Glutamic acid and methionine	1.4	482	1	<i>me</i>
					481	+
1:1	16	Histidine and glutamic acid	19	168	9	<i>hist</i>
					7	<i>pigm</i>
					136	+
1:1	16	Methionine and proline	4.6	300	34	<i>pr</i>
					2	<i>pr me</i>
					264	+

\* The substances were added in the optimal concentration as indicated in the text.

† The test of colonies was carried out by streaking the spores of the colonies from supplemented MM on to MM, and isolating streaks which did not grow.

‡ The symbol of the mutant characters stands for the phenotype (see Table 1, note \*).

Table 3 gives the detail of the experiments. The strains isolated included three kinds of recombinants: (a) those with all the 'wild' characters of the original strains; (b) those with some of the 'wild' characters of one strain and one of the 'mutant' characters of the other strain; (c) those with 'mutant' characters from both strains.

As a check on the possible presence of recombinants with more than two requirements, a mixed culture 51 days old (MM:CM=2.3:1) was plated on CM. From each of 270 colonies point-transferences were made to MM supplemented by both the requirements of one of the parents, and to MM supplemented by both the requirements of the other; 47 (17 %) of the colonies grew on both media. All the others grew on either one or the other. Thus no strain with more than two deficiencies was detected in this preliminary test.

## DISCUSSION

This work is still in a preliminary stage, and no genetic conclusions can be drawn. The precautions taken and the controls carried out are sufficient to exclude the attribution of the appearance of new types to any processes independent of the interaction of the different strains, such as back-mutation

or adaptation. The direct evidence for the interaction between two strains with complementary nutritional requirements is striking enough: see Pl. 1, figs. 1 and 2, for one example of this and Pl. 2, fig. 5, for another. Selection of prototrophic clones from mixed cultures has been amply demonstrated. The stability of some of these clones for two or three subcultures has been checked. It is thus legitimate to conclude that the appearance of new stable prototrophic clones is the result of the interaction of two auxotrophic clones. The negative result obtained on plating together spores of strains 5 and 14 on MM indicates that the two strains require a period of common growth before they will give rise to recombinants. The clearest evidence for the occurrence of recombination is the finding of 51 strains carrying one 'mutant' character from one of the two strains and 'wild' characters from the other, and especially the finding of two strains with one 'mutant' character from one of the two strains and one 'mutant' character from the other.

The authors wish to express their thanks to Dr A. Tonolo for his collaboration in the first part of this work; also to Mr F. G. Miles for the English translation of the Italian text, and to Mr A. Piccirilli for the photographs.

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## EXPLANATION OF PLATES

## PLATE 1

Fig. 1. Syntrophic tufts on CM agar between colonies of strains *3 hist* (dark colonies) and *4 un pigm* (light colonies). About natural size.

Fig. 2. Enlarged detail of fig. 1.

## PLATE 2

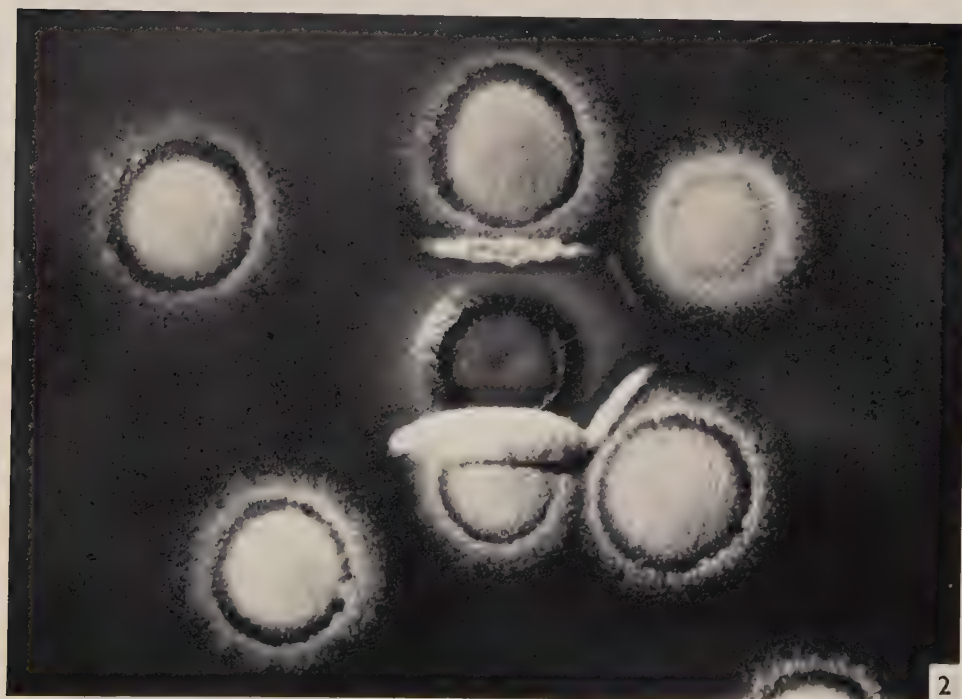
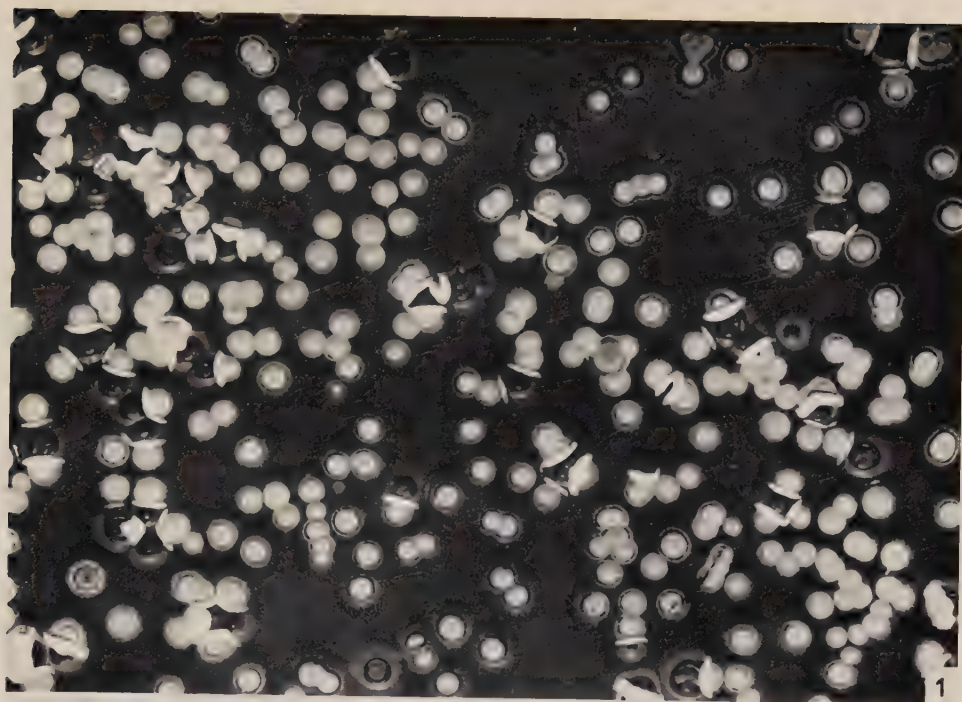
Fig. 3. Prototrophic sectors which have overgrown a colony derived from a syntrophic tuft transferred to MM agar (strains *5 me hist* and *14 pr glu pigm*). Magnification about  $\times 7$ .

Fig. 4. Growth on limiting agar slopes (MM:CM=1:1) of strains *5 (me hist)* and *14 (pr glu pigm)* separately and in mixed culture *5+14*. Note exuberant growth of the mixed culture, and dark pigment diffused into the agar.

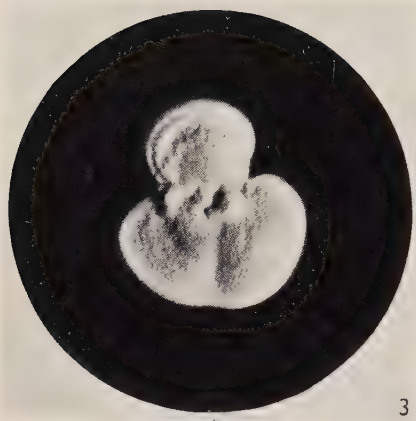
Fig. 5. Growth on MM agar of prototrophic recombinants after plating  $1.4 \times 10^6$  spores from a limiting agar (MM:CM=1:1) mixed culture of strains *5 me hist* and *14 pr glu pigm*.

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G. SERMONTI & I. SPADA-SERMONTI—RECOMBINATION IN STREPTOMYCES. PLATE 1  
(Facing p. 616)



G. SERMONTI & I. SPADA-SERMONTI—RECOMBINATION IN *STREPTOMYCES*. PLATE 2

VAN WAGTENDONK, W. J., VAN TIJN, B., LITMAN, R., REISNER, A. & YOUNG, M. L.  
(1956). *J. gen. Microbiol.* **15**, 617-619

## The Surface Antigens of *Paramecium aurelia*

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**SUMMARY:** A surface antigen preparation obtained from *Paramecium aurelia* absorbs from a concentrated antiserum approximately 74 % of the total antibody absorbed by the intact animal. An antiserum prepared against this preparation immobilized *P. aurelia* of the same serotype.

Antisera against the various serotypes of *Paramecium aurelia* are prepared by injecting homogenates of mechanically disrupted organisms into a rabbit (Sonneborn, 1950). These antisera contain antibodies against antigens other than the immobilization antigen (van Wagtendonk & van Tijn, 1953). In an effort to obtain partially purified preparations of the immobilization antigen, preparatory to the determination of its chemical constitution, a method was devised to obtain the antigen without disrupting the cell. This antigen preparation which includes the trichocysts, but not the cilia, will be referred to as the 'surface antigen preparation'. The properties of this preparation are described in this communication.

### RESULTS

*Paramecium aurelia*, var. 4, stock 51.7, serotype A was grown in a bacterized lettuce extract medium. Several litres of a 24 hr. old culture were first concentrated by filtration through a Berkefeld filter. The organisms were then concentrated and partially freed from bacteria by electromigration through a sterile salt solution (van Wagtendonk, Simonsen & Zill, 1952). The volume of the concentrated organisms was carefully measured and the number of *P. aurelia* present determined by a count of samples of a suitable dilution. Molar NaCl solution was added to the suspension to reach a final concentration of 0.06 M. The mixture was gently shaken for 1 min. and centrifuged for 2 min. at 600g. The supernatant fluid, containing the trichocysts (microscopical examination showed the absence of cilia) was carefully decanted and saved. The sediment was resuspended in 0.06 M-NaCl solution and treated in the same way. The two supernatant suspensions were combined, centrifuged for 1 hr. at 2000g in a refrigerated centrifuge, dialysed for 24 hr. against running distilled water and finally concentrated by lyophilization.

The number of organisms present after the first treatment and after the second treatment was determined by a count of the organisms present in a suitable dilution and the loss by lysis calculated. It can be seen from Table 1

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that no lysis took place during the first salt treatment and that the loss due to lysis during the second treatment varied from 2 to 9 %, with a mean of 5.5 %.

The lyophilized preparations were taken up in 10 ml. of a salt solution according to Ringer (diluted 1:10, v/v) and 1 ml. portions were incubated with 1 ml. of 51A antiserum (diluted 1:2, v/v with diluted salt solution according to Ringer) for 1 hr. at 32° and left standing at 4° for 24 hr. The precipitate was centrifuged, washed once with the diluted salt solution and nitrogen determined using the micro-Kjeldahl method and the digestion mixture of Brüel, Holter, Linderstrom-Lang & Rozits (1947). The nitrogen content of the surface-antigen preparation was also determined.

Table 1. *Effect of salt treatment on the lysis of Paramecium aurelia*

No. of organisms before salt treatment	No. of organisms after 1st treatment	No. of organisms after 2nd treatment	Percentage lysis
12,800	12,800	12,300	3.9
13,500	13,500	13,150	3.0
39,700	39,700	36,100	9.1
37,000	37,000	34,400	7.0
174,000	174,000	159,000	8.6
106,000	106,000	97,000	6.6
93,000	93,000	91,000	2.2
120,000	120,000	116,000	3.3
			Mean 5.5

Incubation of the antigen preparation with antisera prepared against homogenates of serotypes 51B and 51D did not result in the formation of a precipitate. Furthermore, the antigen preparation formed only one ring when incubated with 51A antiserum according to Oudin (1948).

Untreated *Paramecium aurelia*, serotype A, were incubated with the same serum and the amount of antibody absorbed determined. It can be seen from Table 2 that the absorption of antibody by whole organisms from a concentrated antiserum is only slightly greater than the absorption by the surface antigen preparation.

Table 2. *Absorption of antibody from an antiserum prepared against a homogenate of Paramecium aurelia, var. 4, serotype 51A by various preparations obtained from P. aurelia*

Preparation	No. of <i>P. aurelia</i> × 10 <sup>5</sup>	Total N in preparation (μg.)	N per organism (μg. × 10 <sup>5</sup> )	Total N absorbed (μg.)	N absorbed per organism (μg. × 10 <sup>5</sup> )
Live	17.4	350.4	201	34.5	20
	12.0	266.3	222	55.0	46
	13.5	315.2	233	34.9	26
	12.0	298.3	249	32.8	27
	Mean				29.7
Surface antigen	15.9	9.2	5.8	24.0	15
	9.1	8.6	9.5	44.1	48
	11.6	8.6	7.4	34.0	29
	12.0	5.2	4.3	28.2	24
	Mean				29.0
Supernatant of homogenate	14.0	226.8	162	72.1	52



Finally, surface-antigen preparations were injected into a rabbit and an antiserum prepared. This antiserum immobilized *Paramecium aurelia*, serotype A, in a dilution of 1 : 200 after 2 hr.

#### DISCUSSION

The experiments demonstrate that the surface antigen preparation from *Paramecium aurelia*, serotype 51A, does absorb antibodies from a serum prepared against homogenates of the same type, and that an antiserum prepared against the surface antigen preparation does react specifically with organisms of the same serotype in causing immobilization of the organisms. The fact that the Oudin test shows only a single band indicates that the preparation may contain a single antigenic substance. It must be pointed out that the preparation contains trichocysts and any soluble surface substances associated with the organism. Whether the antigenic activity of the preparation should be ascribed to the trichocysts, to soluble substances, or to both, is not known.

A correction can be made in the value for the absorption of antibodies by the antigen preparation. If it be assumed that 10 % of the originally present organisms lysed, these animals could have contributed to the absorption value of the preparation. In an earlier publication (van Wagtendonk & van Tijn, 1953) the absorption by a supernatant fluid from a homogenate of *Paramecium aurelia* was determined. This value is given in Table 2. The supernatant fluid of 14,000 lysed *P. aurelia* would contribute  $7 \times 10^{-5}$   $\mu\text{g.}$  of absorbed nitrogen to the determined value for the absorption by the surface antigen preparation. Thus the surface antigen prepared from one *Paramecium* would absorb  $22.0 \times 10^{-5}$   $\mu\text{g.}$  or approximately 74 % of the total antibody absorbed by the intact organism.

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## Serological Relationship between Potato Paracrinkle Virus, Potato Virus S and Carnation Latent Virus

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**SUMMARY:** Evidence is given that potato paracrinkle virus, potato virus S and carnation latent virus are serologically related and should be considered as related virus strains, although they differ considerably in their host range and pathogenicity, and only carnation latent virus is transmissible by aphids. It is suggested that the three have evolved from a common aphid-transmitted ancestor. In addition to the antigens which the three have in common, each has many specific ones, and the two strains from potato are more closely related to one another than to the carnation virus. No plant of the potato variety King Edward was found free from paracrinkle virus, and no Arran Victory plant free from virus S. Minor variants of both paracrinkle virus and of virus S were detected; it is suggested that the variations in severity of symptoms developed when Arran Victory plants are grafted with King Edward scions reflect the various degrees to which different isolates interfere with each other's multiplication. Most isolates of virus S interfere only slightly with the multiplication of paracrinkle virus.

Salaman & Le Pelley (1930) found that plants of the potato variety Arran Victory became diseased when grafted with scions from apparently normal plants of the variety King Edward. They gave the name paracrinkle to the disease in Arran Victory plants, and since their original observation every plant of the variety King Edward which has been tested has been found to contain paracrinkle virus. This virus has not been found to occur naturally in any other variety of potato or in any other plant, and it has featured frequently in speculations about the origin of viruses, usually with the suggestion that it might be an intrinsic component of the original King Edward seedling (Bawden, 1939; Darlington, 1944, 1949; van der Plank, 1948). For long it was thought to be transmissible only by grafting, but Bawden, Kassanis & Nixon (1950) showed that it could be transmitted by mechanical inoculation of sap, particularly to tomato, and that infected plants contained characteristic elongated particles.

While studying viruses which occur in the carnation (*Dianthus caryophyllus* L.), I found one, which I called carnation latent virus, that had particles apparently identical with those found in tomato plants infected with paracrinkle virus. Plants infected with paracrinkle virus also reacted specifically with antisera prepared against carnation latent virus. It then seemed that paracrinkle and carnation latent virus might be serologically related, that is, were strains of one type virus. But this idea later appeared untenable because I found that apparently healthy plants of many other potato varieties also contained a virus which reacted specifically with antisera prepared against carnation latent virus. Not only were these other potato varieties free from

paracrinkle virus, but many of them, such as Arran Victory, became diseased when infected with it. It seemed probable that these varieties contained potato virus S, which Ouboter (1951) and Rozendaal (1952) described as prevalent in apparently healthy plants of many Dutch potato varieties. That this was so was shown by tests with an antiserum against potato virus S kindly provided by Professor E. van Slogteren (Laboratory for Flowerbulb Research, Lisse, Holland). This antiserum also precipitated specifically with sap from King Edward plants and with sap from plants infected with carnation latent virus. Thus it was clear that carnation latent virus and potato virus S were serologically related to one another; but the results from tests with King Edward had two possible interpretations. One was that paracrinkle virus was serologically related to potato virus S and carnation latent virus; the other that King Edward plants contain not only paracrinkle but also potato virus S. Serologically related viruses are usually mutually antagonistic in plants, and plants systemically infected with one usually resist infection with another. As varieties like Arran Victory are infected with virus S and yet readily succumb to infection with paracrinkle virus, paracrinkle and S viruses seemed unlikely to be related, and I suggested (Kassanis, 1955) that King Edward potatoes contained paracrinkle and virus S and that the elongated particles depicted by Bawden *et al.* (1950) were probably virus S and not paracrinkle. The further work I have now done, and which is described below, has led me to revise this opinion and to conclude that apparently healthy plants of King Edward potato contain only one virus, that of paracrinkle, which, as also suggested by Wetter & Brandes (1956), is related to potato virus S.

#### METHODS

Carnation latent virus was propagated in Sweet William plants (*Dianthus barbatus* L. var. Sutton's Scarlet). Tubers of King Edward and Arran Victory, free from all known viruses except potato paracrinkle and potato S, were kindly supplied by Dr G. Cockerham. Many tubers of these two varieties from different stocks were also tested in an unsuccessful attempt to find virus-free stocks. Seeds of *Solanum demissum* Lindl. (Commonwealth Potato Collection nos. 2167 and 2168) were kindly supplied by Dr W. R. S. Wortley and tubers of the Dutch variety Profijt by Dr Rozendaal.

To facilitate mechanical transmission 'Celite' was always added to the inoculum. Sap from Sweet William plants contains an inhibitor which prevents the mechanical transmission of carnation latent virus from carnation to other species of plants, therefore this virus was transmitted by the aphid *Myzus persicae* Sulz. A larger proportion of aphids transmitted when these were left without food for 2-4 hr. before feeding for a few minutes on the infected plants than when they fed for long periods on the infected plants. Infective aphids transmitted the virus only to the first test plant when a series of plants was colonized at hourly intervals. The virus is not readily transmitted by aphids and healthy plants were therefore colonized with 15-20 aphids/plant; with Sweet William these conditions usually infected 50 % of the plants. Many



attempts to transmit a virus by aphids from King Edward or Arran Victory potato plants, under a variety of conditions, all failed.

Paracrinkle virus was detected by grafting on to Arran Victory potato plants, and the other viruses were detected serologically. All tests for potato virus S in King Edward, or in other plants infected from it, were made with antiserum prepared against carnation latent virus, to avoid the possibility of confusing reaction with paracrinkle virus, should this be unrelated to potato virus S.

Antisera to the viruses carried by King Edward and Arran Victory potato plants were produced by injecting rabbits with partially purified virus preparations, made either by sedimentation in the ultracentrifuge or by precipitation with ammonium sulphate. The rabbits were bled and the sera separated after six intravenous injections of 2.5 ml. of virus preparations, given at 3-day intervals. The antisera prepared against carnation latent virus antiserum did not precipitate with sap from healthy Sweet William plants, and the antisera against the potato viruses did not precipitate with sap from healthy potato plants, variety Majestic. The serological tests were made as previously described (Kassanis, 1955); unless otherwise mentioned the antisera were diluted to 1/40. For brevity, the antisera prepared against the viruses present in the two potato varieties will be referred to as antiserum against King Edward and antiserum against Arran Victory.

For cross-absorption tests antisera and centrifuged sap from plants were mixed and left at room temperature for 24 hr. after which the mixtures were heated for 10 min. at 60° and centrifuged. This heating did not affect the titre of the unabsorbed antibodies but removed normal plant proteins.

## RESULTS

### *Host ranges and symptoms*

Sap from King Edward potato plants was inoculated to various types of plants; infection occurred in tomato (*Lycopersicum esculentum* L. var. Kondine Red), potato (*Solanum tuberosum* L. var. Majestic and Profijt; *S. demissum*), and sugar beet (*Beta vulgaris* L. var. Kleinwanzleben E.). Except in sugar beet, the infections became systemic. All inoculated plants were tested for their ability to precipitate with carnation latent virus antiserum and for their ability to cause paracrinkle when grafted to Arran Victory potatoes. The second test could not be done directly from sugar beet, but sap from the inoculated leaves was inoculated to tomato plants, scions from which were later grafted to potato plants. Every plant whose sap precipitated with the carnation latent virus antiserum also caused paracrinkle, and the transmissions provided no evidence that King Edward potatoes contain two viruses. If they do contain two viruses then the two have similar host ranges.

Of the plants infected by inoculating sap from King Edward, only sugar beet was also infected by carnation latent virus, and even in this host the two viruses behaved differently, for carnation latent virus invaded plants systemically.



Tomato plants seem to be susceptible to some strains of potato virus S (Köhler, 1955) but not to others (Rozendaal & Brust, 1955). In my tests tomato plants were very readily infected when inoculated with sap from King Edward, but they remained uninfected with virus S from Arran Victory, even when grafted with scions from this plant.

Two types of *Solanum demissum* became infected when inoculated with sap from Arran Victory and King Edward; but whereas type 2167 showed no symptoms, type 2168 developed a blotchy mottle and crinkling when inoculated from King Edward and a general chlorosis from Arran Victory. The symptoms were not considered reliable for the diagnosis of potato virus S, because uninoculated plants of type 2168 also showed somewhat similar symptoms, although serological tests confirmed that they were free from potato virus S.

The potato variety Profijt is used in Holland as an indicator plant to test for potato virus S. I found it a very useful plant in demonstrating that paracrinkle virus and potato virus S can multiply in the same plant, apparently without interfering with each other. Profijt plants inoculated separately with sap from King Edward or Arran Victory, or with a mixture of the two, showed no symptoms in the first year. In the next two years, the progenies from the plants inoculated with sap from Arran Victory again remained apparently healthy, but those infected from King Edward, or with the mixed inoculum, developed a mild but definite mottle. Serological tests with the three antisera gave identical results in both years. The plants infected from King Edward did not react with Arran Victory antiserum at a dilution 1/20, but did so with the antisera to King Edward and carnation latent virus. As the plants which were infected with mixed inoculum developed symptoms, reacted with the Arran Victory antiserum and produced paracrinkle when grafted to Arran Victory potato plants, it can be concluded that they were infected with paracrinkle and potato S from Arran Victory.

The severity of the blotchy mottle and crinkling of the leaves shown by Arran Victory plants infected with paracrinkle virus varies considerably in different plants. This was first stressed by Salaman & Le Pelley (1930) and again by Bawden *et al.* (1950). Variations have been observed even when Arran Victory plants have been grafted with scions taken from the same King Edward plant. It cannot be attributed to growing conditions or age of the infected plants, because the progenies of one Arran Victory plant which showed mild symptoms were followed through three growing seasons and the symptoms remained unchanged.

#### *Properties in vitro*

If King Edward contained paracrinkle virus and virus S, it seemed unlikely the two would both have the same resistance to heating and the same dilution end-point. Attempts to separate the two, however, all failed. The temperature at which potato virus S has been reported to be inactivated *in vitro* varies, depending possibly on the strain but more likely on the method used for testing. Köhler (1955) gave a value of 68°–71°, Rozendaal & Brust (1955) 50°–60°, Wetter & Brandes (1956) 60°–65°, and Levieil (1954) less than 45°. In

my tests 5 ml. samples of sap from King Edward plants were heated for 10 min. at different temperatures and inoculated to tomato plants. A month later these were tested serologically with the carnation latent virus antiserum and also grafted to Arran Victory. The two tests gave identical results. In three experiments of this kind, using a total of 12 tomato plants per treatment, the inactivation end-point was between 80° and 85°. This is 20° higher than the thermal inactivation point found for carnation latent virus (Kassanis, 1955), with which tests were made in sap from Sweet William plants. It is also 20° higher than that reported by Bawden *et al.* (1950), who examined heated sap from tomato plants infected with potato paracrinkle virus by means of the electron microscope and did not detect particles in sap heated for 10 min. between 55 and 60°. I obtained yet another value when sap from King Edward was heated at different temperatures and then tested serologically. Unheated sap and sap heated at 45°, 50°, 55°, 60° or 65° for 10 min. gave precipitation end-points the reciprocals of which were 32, 8, 4, 2, 1 and 0, respectively. Hence the method of testing seems important; insensitive methods record a much lower thermal inactivation point than methods such as inoculation to tomato, which presumably detects small quantities of virus. The serological test suggests that much of the virus is lost at temperatures far below the temperature required to inactivate all particles. The virus also rapidly inactivates when sap is kept at 20°, the serological activity disappearing in 24 hr.; at 35° the precipitation end-point fell from 1/32 to 1/2 in 4 hr.

When tomato plants were inoculated with King Edward sap at various dilutions, there was again complete agreement between tests made serologically for potato virus S or by grafting to Arran Victory for paracrinkle. The number of tomato plants infected out of 4 were 4, 4, 3 and 0, respectively, with undiluted sap and dilutions of 1/10, 1/100 and 1/1000. When, instead of tomato, the potato variety Majestic was inoculated there were 4, 4 and 0 infections out of 4 with undiluted sap and dilutions 1/10 and 1/100. The prevalence of potato virus S in different European and American potato varieties suggests that some strains of this virus other than that present in King Edward might be more infectious. When sap from Arran Victory was tested on plants of the variety Majestic it infected the plants at dilution 1/10 but not at 1/100. Köhler (1955) gave the dilution end-point for potato S as between 1/1000 and 1/10,000. As with thermal inactivation, there is no agreement in the results obtained with different strains of potato virus S.

#### *Serological tests*

After sap from any of the three plants (King Edward, Arran Victory and Sweet William infected with carnation latent virus) was mixed with antiserum prepared against any of the other two, no antigen remained that could be precipitated with the homologous antiserum. Therefore, if paracrinkle virus produces antibodies and precipitates with them, it must be serologically related to potato virus S and carnation latent virus. If serological relationship be taken as a criterion that certain viruses are strains of the same virus, then potato virus S, carnation latent virus and paracrinkle virus should be strains

of the same virus. The possibility remains, however, that paracrinkle virus did not produce antibodies or precipitate with them. If this were so, King Edward might contain a virus related to potato virus S and carnation latent virus, and also to paracrinkle virus which was entirely unrelated to them.

That potato paracrinkle virus is unrelated to potato virus S seems unlikely, for it is easy to show that potato paracrinkle virus is precipitated by the antiserum to carnation latent virus. In one test 2.5 ml. of King Edward sap was mixed with 0.5 ml. carnation latent virus antiserum; after 2 hr. the precipitate was centrifuged down and resuspended in 2.5 ml. water. The suspension was inoculated, undiluted and at dilution 1/10, to 5 tomato plants each. Tests made later on the inoculated plants showed not only that they contained an antigen related to carnation latent virus antiserum, but that they also caused paracrinkle when grafted to Arran Victory potato plants.

Although all my results suggest that paracrinkle, potato S and carnation latent virus are serologically related, they also show that these three viruses are far from being antigenically identical. Table 1 shows that the precipitation end-points of the antisera to carnation latent virus and to King Edward were

Table 1. *Precipitation titres of the antisera to carnation latent virus and King Edward titrated against homologous and heterologous antigens*

Antisera to	Infected Sweet William sap (dilution 1/10)	King Edward sap (dilution 1/4)
	Titre of antisera	
Carnation latent virus	1/1280	1/80
King Edward	1/10	1/320

much higher when titrated against sap containing homologous viruses than when tested against the heterologous viruses at the same concentrations. These, and similar results obtained when the antisera to carnation latent virus and Arran Victory were compared, suggest that the three viruses have only some antigenic groups in common. This is better shown in Table 2, which gives the results of tests on which antisera were absorbed with the heterologous antigens. The results suggest that carnation latent virus and the virus in King Edward probably have much less than half of their antigenic groups in common. The viruses from King Edward and Arran Victory are more similar and probably have about half of their antigenic groups in common.

King Edward antiserum when titrated against sap from Arran Victory always reacted at dilutions up to 1/40, but Arran Victory antiserum did not always react at these dilutions when titrated against sap from King Edward. In one typical test sap from 5 plants of the variety King Edward of the same age and appearance, but raised from tubers collected from different parts of the country, were titrated against Arran Victory antiserum at 1/20. The respective precipitation titres were: 0, 1/2, 1/16, 1/16, 1/20; but all plants reacted at 1/32 with the antiserum to King Edward. The plant which did not react with Arran Victory antiserum at 1/20 did react when the antiserum was used at 1/4.



The results indicate that the different King Edward plants contained virus strains with different degrees of relationship to the strain of virus S used to produce the Arran Victory antiserum. That the strains of virus in different stocks of King Edward differ was also shown by cross-absorption tests. When the King Edward antiserum was absorbed with sap from a line of King Edward different from that used in preparing the antiserum, the absorbed serum still precipitated strongly with sap from the homologous line of King Edward, though it no longer precipitated with the line used for the absorption.

Table 2. *Precipitation titres of absorbed and unabsorbed antisera to King Edward and carnation latent virus titrated against homologous and heterologous viruses*

Antisera to		Infected Sweet William sap (dilution 1/10)	King Edward sap (dilution 1/4)	Arran Victory sap (dilution 1/4)
King Edward	Absorbed with Sweet William sap*	0 (1/2)†	1/320	—
	Unabsorbed	1/8	1/320	—
King Edward	Absorbed with Arran Victory sap‡	—	1/320	0 (1/20)
	Unabsorbed	—	1/640	1/320
Carnation latent virus	Absorbed with King Edward sap§	1/1280	0 (1/20)	—
	Unabsorbed	1/1280	1/80	—

\* 2 ml. antiserum + 2 ml. of sap from Sweet William plants infected with carnation latent virus.

† Figures in brackets show the dilution at which the titration was started.

‡ 0.3 ml. antisera + 5.7 ml. of sap from Arran Victory plants.

§ 0.5 ml. antisera + 9.5 ml. of a preparation of virus from sap of King Edward plants, concentrated 5 times by precipitation with ammonium sulphate.

## DISCUSSION

As it is impossible to prove a negative, so it is impossible to be sure that King Edward plants do not contain both paracrinkle virus and a virus which is related to potato virus S and carnation latent virus. However, all my attempts to demonstrate a dual infection have failed, and until some evidence is found for this, it seems reasonable to assume that paracrinkle virus is serologically related to the much more widely distributed potato virus S and carnation latent virus. This conclusion raises problems of nomenclature, for paracrinkle virus was the first to be discovered and named, and so the others should perhaps be regarded as strains of it and renamed appropriately.

There is nothing unusual in serologically related strains of a virus differing in their host ranges, pathogenicity and method of transmission to the extent that paracrinkle, S and carnation latent viruses differ from one another. Indeed, the only reason for doubting that paracrinkle virus and virus S are not related strains is that plants infected with virus S are still susceptible to infection by paracrinkle virus, for serologically related viruses are usually mutually antagonistic and plants systemically infected with one usually resist invasion by another. However, here again this behaviour is not unprecedented; Bawden



& Kassanis (1951) found two strains of potato virus Y which did not interfere with one another when multiplying in the same plant. Also, Matthews (1949) found with potato virus X that the extent to which infection with one strain protected plants against infection by another depended on the degree of serological relationship between them. My results suggest that paracrinkle virus and potato virus S each has many specific antigenic groups, and, by analogy with the results with potato virus X, it is therefore not unexpected that they should fail to protect plants against one another. Perhaps they do interfere somewhat with one another and the extent to which they do this reflects the degree of their structural similarity. Different isolates of paracrinkle virus and different isolates of potato virus S seem to differ from one another and, as already stated, the symptoms of paracrinkle produced in Arran Victory plants by transmission even from the same King Edward plant can vary considerably. Salaman (1932) interpreted this variation as evidence that paracrinkle is caused by the combined action of two viruses which occasionally dissociate and then produce less severe symptoms. There is, though, no evidence for such a dual infection, and certainly not for the presence of potato virus Y, as postulated by Salaman; a much more likely explanation is that the severity of symptoms reflects the extent to which the particular form of virus S already present in the Arran Victory plant interferes with the establishment of the form of paracrinkle virus present in the infecting King Edward plant.

The conclusion that paracrinkle virus, virus S and carnation latent virus are related to one another provides an explanation for the universal occurrence of paracrinkle virus in King Edward potatoes without need to postulate that it was a product of the original seedling. It seems likely that all three viruses have originated from a common ancestor which was aphid-transmitted, but that only one line of descendants, represented by carnation latent virus, now retains this character. There are many recent examples of viruses acquiring or losing a character as a result of invading a new host, or after a prolonged sojourn in a new host, and these include examples of losing or acquiring the ability to be transmitted by a given insect (Black, 1953; Hollings, 1955; Watson, 1956). And in a vegetatively propagated plant like the potato, loss of insect-transmissibility would not lead to the disappearance of the virus as it might in annual plants coming from seed, for the virus is perpetuated through the tubers. To survive for long this way in commercial potato varieties, however, a virus must not cause obvious symptoms. Hence, if paracrinkle virus ever existed in other varieties, it has been eliminated because of the disease it caused in them, but in King Edward there has been no selection against it.

I am indebted to Miss Sally Eisler for technical assistance.

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## A Selective Medium for *Brucella* spp.

By E. J. MORRIS

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**SUMMARY:** A selective medium for *Brucella* spp. is described which contains 5-nitrofurfurylmethyl ether, bacitracin, polymyxin and Actidione. Organisms normally encountered in culture from faeces or soil are completely suppressed on the medium while brucellas grow quantitatively within 65 hr. of incubation.

A selective medium is essential when the detection of brucellas by direct culture is required from such material as milk, soil, faeces, etc. Felsenfeld *et al.* (1951) devised a medium containing circulin, polymyxin, bacitracin and sulphadiazine to isolate brucellas from chicken faeces. Kuzdas & Morse (1953), using a similar medium, found that 5 days of incubation were necessary to grow these organisms from soil suspensions. Mair (1955) used polymyxin, penicillin and Gentian violet to detect *Brucella abortus* from herd samples of milk, but reported that the medium was sometimes overgrown by contaminant organisms.

The high antibacterial activity of nitrofurans has been reported by many workers (e.g. Dodd & Stillman, 1944; Paul & Bender, 1950; Dodd, Cramer & Ward, 1950). In the present study it was found that 5-nitrofurfurylmethyl ether incorporated in nutrient agar allowed the quantitative growth of *Brucella* spp., while it inhibited the growth of the majority of other Gram-negative organisms, including *Proteus* spp. The medium finally devised enables brucellas to be diagnosed within a 65 hr. incubation period by direct cultivation from material heavily contaminated with other bacteria.

### METHODS

**Media.** The growth of the brucellas was compared on several basal media, e.g. Difco tryptose agar, Albimi agar, 'Oxoid' nutrient agar and a modified Hartley digest agar (TMA) used in this department. Tryptose agar and TMA were identical in their ability to support growth of brucellas in higher concentrations of the inhibitory constituents of the medium than the other basal media tested. The selective medium finally devised consisted of TMA or Tryptose agar at pH 7.4 with 1/30,000 (v/v) 5-nitrofurfurylmethyl ether ('Furaspor'; Menley and James Ltd.); 25 units bacitracin/ml.; 4 units polymyxin B ('Aerosporin'; Burroughs Wellcome and Co.)/ml. and 1/10,000 (w/v) Actidione (The Upjohn Co. U.S.A.). The additions were made to the melted agar cooled to 50° and poured into Petri plates in 20 ml. amounts. The agar plates were dried for 2 hr. at 37° to remove surface moisture and could be stored at 4° for at least 4 weeks without loss in selective properties. The medium was equally selective when bacitracin was replaced by licheniformin.

5-Nitrofurfurylmethyl ether is a viscous yellow liquid soluble at 1/500 in water at room temperature. It is heat labile and completely inactivated by autoclaving at 120°. A 1/500 aqueous solution remains active after several months of storage in the dark at 4°.

*Growth tests.* The Miles & Misra (1938) technique was used to inoculate the control and test media with pure cultures of brucellas and common contaminating organisms. Inocula were adjusted to give *c.* 200 colonies of brucellas/plate and 2000 of other organisms per plate. *Brucella* spp. were also added to suspensions in water of soil (1 g./20 ml.) and faeces (1 g./100 ml.) so that 0.1 ml. when plated on the media would yield *c.* 100 brucella colonies. Tryptose agar and TMA were used as control media. Incubation for all tests was at 37° for 65 hr. in air and in air + 10 % (v/v) CO<sub>2</sub>.

## RESULTS

*Growth of pure cultures.* The 19 strains of the genus *Brucella* examined (*Brucella suis* (6), *B. abortus* (8), *B. melitensis* (3) and two intermediate strains) all gave similar counts on the selective nitrofuran medium and control agar. The results of a typical experiment are summarized in Table 1. Colonies were

Table 1. *Colony counts of Brucella species on the nitrofuran selective medium and on control nutrient agar*

Species		Nitrofuran medium	Control agar
		(Colonies/plate)	
<i>Brucella suis</i>	PS 3	290	289
	K/B	198	191
	S/R	269	275
	37	145	158
	B 64	204	208
	B 67	181	221
<i>B. melitensis</i>	MM 6015	212	226
	B 63	141	156
	B 1020	165	178
<i>B. abortus</i>	29	221	198
	B 31	168	157
	B 34	182	186
	B 41	135	136
	B 47	214	225
	B 127	236	221
	B 1019	222	243
	S 19	103	122
Intermediate	B 55	142	154
	B 206	180	175

smaller (0.8–1.0 mm. diam.) on the selective medium than on control agar (1.2 mm. diam.) but were otherwise typical in appearance (Fig. 1). *B. suis* and *B. melitensis* grew equally as well when incubated in air or in the air + CO<sub>2</sub> mixture. Slide-agglutination tests were normal with colonies grown on the selective medium.







E. J. MORRIS—SELECTIVE MEDIUM FOR *BRUCELLA* SPP. PLATE 1

(Facing p. 631)

Growth of the following organisms (showing in parentheses the number of strains tested) was completely inhibited on the selective medium: *Proteus vulgaris* (3), *Escherichia coli* (4), *Aerobacter aerogenes* (2), *Alcaligenes faecalis* (6), *Streptococcus faecalis* (1), *Micrococcus tetragenus* (5), *Staphylococcus aureus* (3), *Pseudomonas aeruginosa* (1), *Serratia marcescens* (2), *Bacillus* spp. (20), *Salmonella* spp. (6), *Achromobacter* spp. (6).

*Tests with soil and faecal suspensions.* In most instances *Brucella* spp. were grown in pure culture on the selective medium from the many soil suspension mixtures tested. Growth of contaminant organisms in each case was confluent on control agar. Colonies which sometimes developed on the medium were mainly *Achromobacter* species; their numbers (2–20/plate) did not interfere with diagnosis of brucellas. *Brucella* spp. were also isolated without difficulty from the artificially infected suspensions of guinea-pig faeces (Fig. 2). On rare occasions, however, contaminant colonies encountered on the medium from faecal suspensions were unusually high (200/plate). The majority of these colonies were *Alcaligenes* spp. and were suppressed when the medium was incubated in an atmosphere of air + 10 % (v/v) CO<sub>2</sub>, while growth of *B. suis* and *B. melitensis* was unaffected by these conditions.

I am indebted to Dr J. A. Olsen of Eaton Laboratories and Dr M. L. Graeme of Menley and James Ltd. for generous gifts of nitrofurans compounds and to Mr L. H. Kent and Dr B. T. Tozer for a supply of licheniformin. Thanks are also due to Professor J. C. Cruickshank and Dr J. E. Smith for brucella cultures and to Mr C. M. R. Pitman for technical assistance.

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#### EXPLANATION OF PLATE

- Fig. 1. Left: colonies of *Brucella suis* on Tryptose agar after 65 hr. incubation at 37° in air. Right: the same inoculum on the selective nitrofurans medium.
- Fig. 2. Left: growth from 0.1 ml. of a faecal suspension on Tryptose agar after 65 hr. incubation at 37° in air. Right: the same inoculum on the selective nitrofurans medium showing a pure growth of *Brucella suis*.

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## Internal Structure of *Rickettsia burnetii* as shown by Electron Microscopy of Thin Sections

By M. G. P. STOKER, K. M. SMITH AND P. FISET

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**SUMMARY:** Thin sections of purified suspensions of *Rickettsia burnetii* were examined by electron microscopy. The organism possesses a limiting membrane, within which lie a granular region and a dense central body. The configuration of the central body suggests that it may consist of an elongated and irregularly twisted strand.

The development of techniques for examining thin sections by electron microscopy has led to studies on the internal structure of several bacteria and viruses. Sections of bacteria show them to be almost filled with dense granular or sponge-like material closely surrounded by a limiting membrane, but with less dense 'vacuoles' in the centre which contain small threads and elongated bodies thought to correspond to a nuclear apparatus (see Chapman & Hillier, 1953; Kellenberger & Ryter, 1955; Bradfield, 1956; Maaløe & Birch-Andersen, 1956). Animal viruses have been examined in thin sections of infected tissue. Vaccinia and fowl-pox viruses are oval with a single or double limiting membrane and an eccentrically placed inner body (Gaylord & Melnick, 1953; Morgan, Ellison, Rose & Moore 1954*b*); herpes virus is round or oval with a central body and one or two membranes probably depending upon the stage of development and the position in the cell (Morgan, Ellison, Rose & Moore 1954*a*); elementary bodies of meningo-pneumonitis virus are dense with central granules, but large circles with no internal granules are also seen in sections of infected tissue (Gaylord, 1954).

The rickettsias are commonly regarded as intermediate between bacteria and the larger animal viruses. Their size, staining properties and metabolic activity link them to the bacteria, but, like viruses, they remain dependent upon living cells for growth. It is therefore of some interest to know how their fine structure resembles those of the viruses and bacteria. Electron microscopy of intact rickettsias has confirmed that they are rod-shaped organisms, about 300 m $\mu$ . in diameter, and varying from 500 m $\mu$ . to at least 1000 m $\mu$ . in length. A limiting membrane may be seen and this is separated from an electron dense central portion which in shadowed preparations shows a convoluted irregular surface and remains elevated above the supporting membrane (Stoker, 1950); but the internal structure cannot be clearly seen when the whole organism is examined in this way. In the studies to be described in this paper the internal structure of *Rickettsia burnetii* was examined by electron microscopy of ultra-thin sections.



## METHODS

*Preparation of suspension of Rickettsia burnetii*

Preparations were made from infected yolk sacs of the second egg passage of a guinea-pig-adapted line of the Nine Mile strain of *Rickettsia burnetii*. The harvested yolk sacs were stored at  $-20^{\circ}$  until used. The purified suspension of rickettsias was made from the infected yolk sacs by ether extraction and differential centrifugation, as for preparation of antigen (Stoker, 1953). This method normally involves treatment with formalin but in the electron microscope preparations osmic acid fixation gave better results, and so formalin was excluded from all suspending media during purification and storage. Judging by electron microscopy, the final preparations were almost completely free from extraneous material. They were stored at  $4^{\circ}$  in phosphate buffered saline up to 2 weeks before fixation and embedding.

*Sectioning technique*

The purified suspensions were centrifuged at 8000 r.p.m. for 30 min. and fixed by resuspension in 2 % osmic acid in citrate phosphate buffer for 2 hr. The fixed rickettsias were then recentrifuged, and the pellet was dehydrated with ethanol in 10 % steps. It was embedded in a mixture of 4 parts *n*-butyl methacrylate and 1 part methyl methacrylate. The sections were not shadowed with metal since the methacrylate was not removed, and they were cut on a Cook and Perkins microtome fitted with a glass knife. Some of the electron micrographs were taken on a Siemens-Elmiskop I microscope at 80 kV., and the others on a Metropolitan Vickers EM 3 electron microscope at 75 kV. Those sections which were thin enough were photographed at a magnification of  $\times 40,000$  on the Siemens microscope and later enlarged optically. Either carbon or formvar supporting films were used in the grids.

## RESULTS

Electron micrographs of the sections of the osmic acid fixed rickettsias are shown in Pls. 1 and 2. The organisms were fairly closely packed, and differed in orientation in various parts of the pellet, so that in some places they were sectioned longitudinally (Pl. 1, fig. 1) and, in others almost transversely (Pl. 1, fig. 2; Pl. 2, fig. 3).

The size of the rickettsias cannot be estimated accurately because of the different planes and angles of section. Assuming that circular sections are transverse, however, it is of some interest that the largest of these sections (which are presumably not at the extremities), show a diameter of less than 200 m $\mu$ . Even allowing for shrinkage in the methacrylate this is considerably smaller than the diameter usually found by conventional electron microscopy of intact organisms, and suggests that the latter have flattened and spread during settling and drying on a surface.

All the organisms showed a distinct limiting membrane of finely granular material 5–10 m $\mu$ . in thickness. The limiting membranes of some of the

rickettsias are fractured but many appear to be intact. Inside the membrane was a clearly defined intermediate zone of moderately dense material surrounding a very dense irregular central body (Pl. 2, fig. 4).

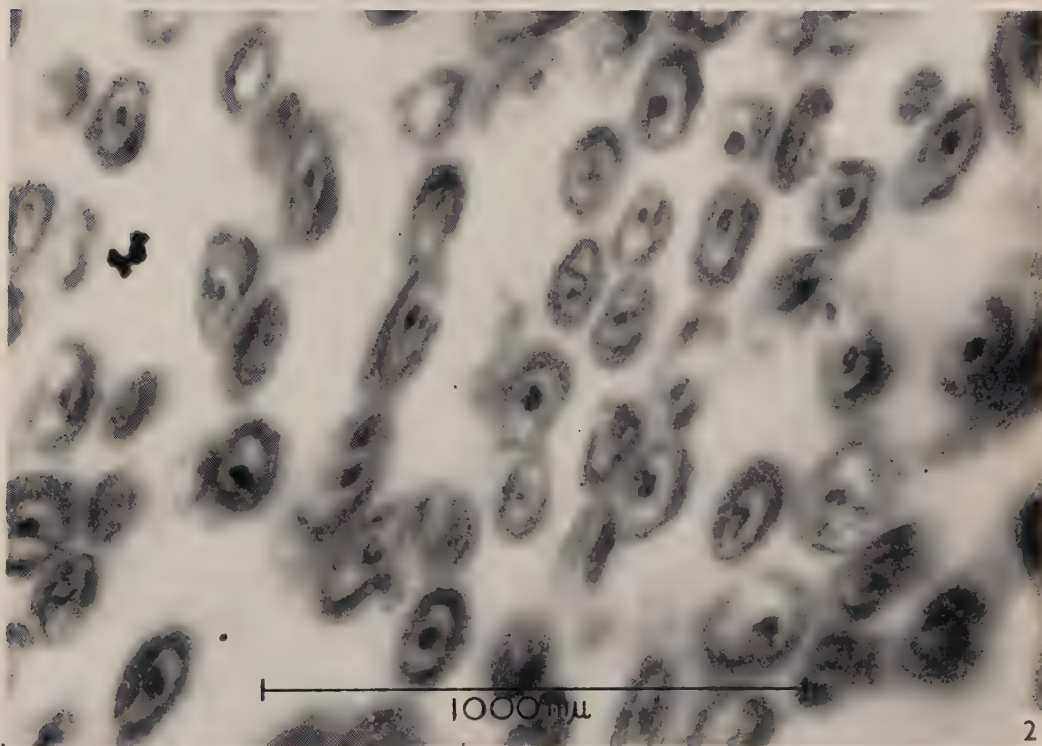
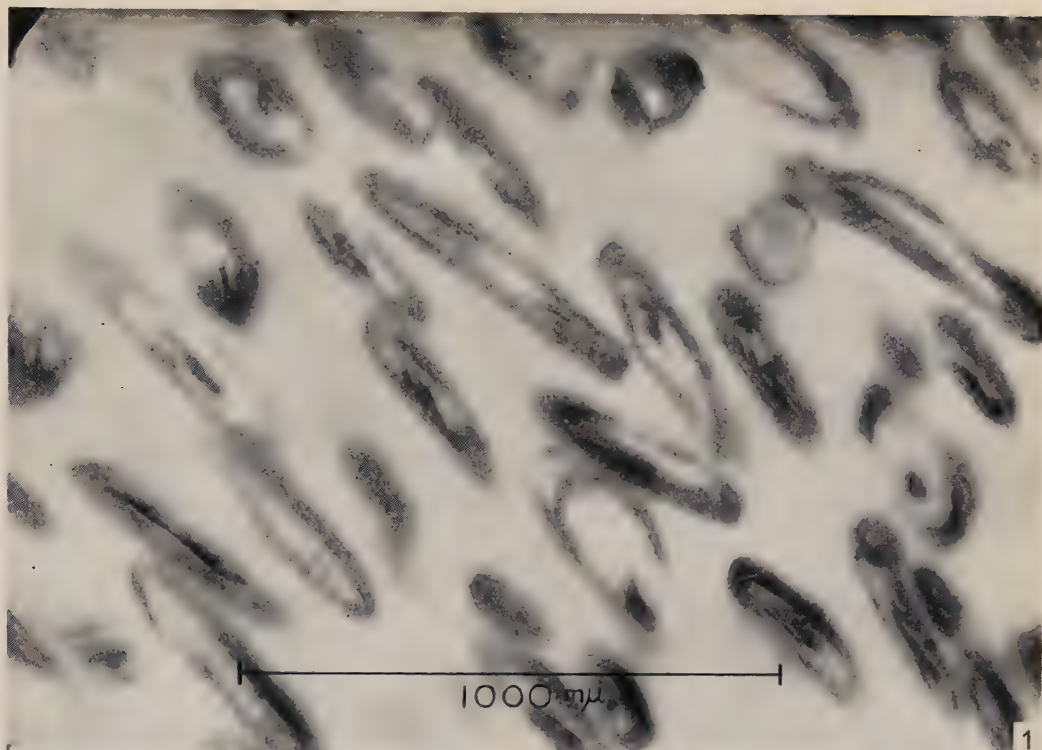
The intermediate zone was somewhat separated from the outer limiting membrane, or its density diminished as it approached the membrane. When not cut obliquely this intermediate zone was about 25 m $\mu$ . in thickness and contained granules of about 5 m $\mu$ . in diameter. The density of the region appears to be less than that of the main granular region of sectioned bacteria, but the granules themselves were of similar size.

Within the intermediate granular region of the rickettsias was a 'hole' in which lay a very dense central body. The latter was seen in most sections of the organisms and presumably therefore extended through a large part of the length of each. The configuration of this central body varied greatly in different organisms. In many it was large, circular and apparently hollow, in some small circular and solid, and in others comma shaped or sigmoid. One, or possibly two, irregularly twisted strands appeared to give rise to this appearance, but serial sections would be needed for confirmation. Pl. 2, fig. 5, however, shows a single rickettsia sectioned longitudinally and in which the central body appears to be a single elongated strand twisted and doubled back on itself.

#### DISCUSSION

From the sections it appears that *Rickettsia burnetii* has a limiting membrane and an intermediate zone of granular material resembling that of many bacteria which have been examined (compare, for example, sections of *Escherichia coli* by Birch-Andersen, Maaløe & Sjöstrand, 1953). There is also a space containing a central body. In the rickettsia, however, the intermediate granular region is far less dense than in bacteria and probably occupies a small proportion of the volume of the organism, while the central body of the rickettsia, on the other hand, is very dense, clearly defined, and proportionately large, compared with the bodies seen in the central region of bacteria.

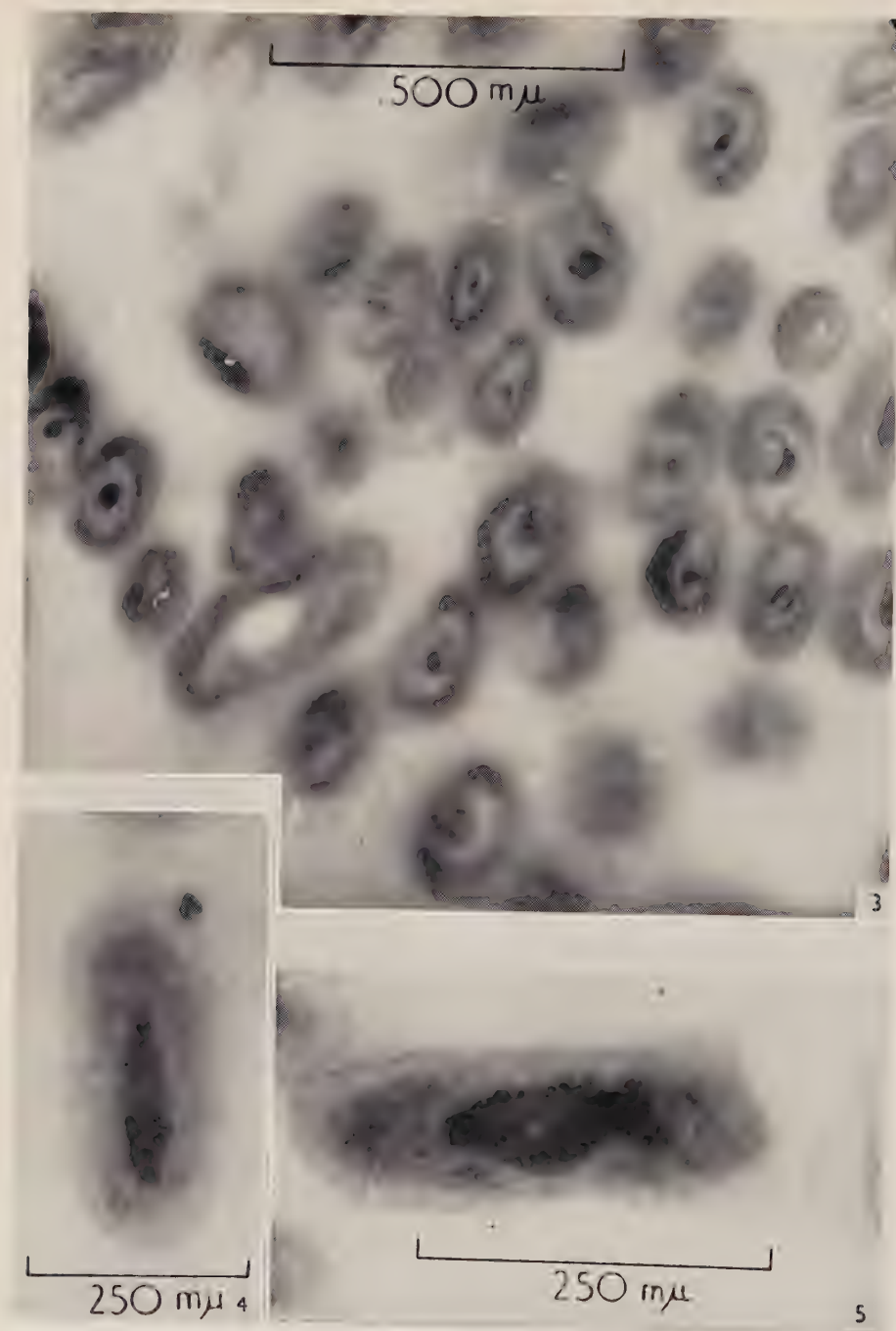
By using a modified Feulgen reaction Bradfield (1954) showed that the deoxyribonucleic acid of staphylococci and paracolon bacilli was situated in the region of the central vacuole. No such studies have been made on *Rickettsia burnetii*, but it is known that the organism has a relatively high content of deoxyribonucleic acid as compared with bacteria (Smith & Stoker, 1951) and it is possible that this is associated with the prominent central body. It would be interesting to know whether the relatively sparse granular region of the rickettsia is in any way connected with their low content of ribosenucleic acid, and, in turn, with the presumed lack of ability of rickettsias to synthesize protein. As may be expected, the structure of the rickettsias in purified suspensions is less variable than that of viruses in the sections of infected tissues showing various stages of development (Morgan *et al.* 1954*a, b*). Nevertheless, sections of vaccinia and herpes viruses show, on a small scale, some particles with a central body and a double membrane not unlike transverse sections of *R. burnetii*.



M. G. P. STOKER, K. M. SMITH AND P. Fiset—INTERNAL STRUCTURE OF *RICKETTSIA BURNETII*. PLATE 1

(Facing p. 634)





M. G. P. STOKER, K. M. SMITH AND P. Fiset—INTERNAL STRUCTURE OF *RICKETTSIA BURNETII*. PLATE 2



We wish to express our gratitude to Miss S. Vernon Smith, of the Agricultural Research Council Virus Research Unit, and Mr R. Horne, of the Cavendish Laboratory, for assistance with the electron micrographs. One of the authors (P. F.) is a Professional Training Fellow of the Department of Health and Welfare of the Province of Quebec.

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## EXPLANATION OF PLATES

## PLATE 1

- Fig. 1. Electron micrograph of purified suspension of *Rickettsia burnetii* with organisms sectioned longitudinally. ( $\times 72,000$ .)
- Fig. 2. Electron micrograph of purified suspension of *Rickettsia burnetii* with organisms sectioned transversely. ( $\times 72,000$ .)

## PLATE 2

- Fig. 3. Electron micrograph of purified suspension of *Rickettsia burnetii* with organisms sectioned transversely. ( $\times 96,000$ .)
- Fig. 4. Electron micrograph of single rickettsia sectioned longitudinally. ( $\times 120,000$ .)
- Fig. 5. Electron micrograph of single rickettsia, sectioned longitudinally. ( $\times 192,000$ .)

(Received 13 June 1956)

## Production of Tryptophan by *Salmonella typhi* and *Escherichia coli*

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**SUMMARY:** In order to determine whether *Salmonella typhi* in growth produces free tryptophan in excess, extracts and cultures were tested by various microbiological methods. A positive result was seldom seen, but when excess indole was added to the culture, comparatively large yields of tryptophan were obtained with all strains examined except one in which there was some disability in the indole  $\rightarrow$  tryptophan process. *Escherichia coli* behaved in the same way, the free tryptophan produced being preserved owing to inhibition of tryptophanase by the indole added.

Logie (1919-20) was the first to show that certain bacteria could synthesize tryptophan. They were grown on a medium in which  $\text{NH}_3$  was the only source of nitrogen and when tested by the glyoxylic method gave a positive reaction for tryptophan. Fildes, Gladstone & Knight (1933) confirmed this result. An extensive literature has since developed on the details of the biosynthesis of tryptophan and its relationship to bacterial nutrition. This need not be discussed. So far as the biosynthetic chain is concerned, it has been proved by chemical and biological methods that anthranilic acid, indole and serine are concerned in the process, but the final product tryptophan has not been determined satisfactorily until recently, except in the case of the mould *Neurospora crassa*. Tatum & Bonner (1944) made short reference to production of tryptophan according to colour tests from indole and serine by *Escherichia coli* suspensions, but Fildes (1945) failed with *Salmonella typhi* and, though Fildes & Rydon (1947) found that 4-methyltryptophan inhibited the further use of tryptophan and so might be expected to cause accumulation of tryptophan in the culture, this did not occur according to colour tests.

During a study of a bacteriophage which required tryptophan for absorption, it became necessary to settle whether free tryptophan was, in fact, present in or on the typhoid bacteria in use. Since, previously, chemical methods of detection had failed, microbiological methods were now used.

Two series of experiments were carried out. In the first the bacteria were extracted with buffer at 100° and the extracts assayed for tryptophan by a microbiological growth method. In the second series use was made of the *Escherichia coli* mutants now available which have an essential requirement for tryptophan in growth. These were used in two ways:

(a) Along the lines of Winkler, van Doorn & Royers (1952) to show that these coli mutants can grow syntrophically with *Salmonella typhi*, although without the symbiosis they can only grow with added tryptophan. The fact

of the growth of the coli mutant syntrophically with another bacterium is taken to imply that the other bacterium has produced free tryptophan.

(b) To produce a 'satellite' effect, i.e. to determine whether a patch of the bacterium under test for production of tryptophan will produce satellite colonies or zones on the surrounding lawn of mutant coli organisms.

#### METHODS

*Organisms used.* *Salmonella typhi*: 'Bact 1', a strain without synthetic block; O 901 R, a rough mutant of the well-known strain O 901, which has a synthetic block immediately below indole; 1808, described later; others as stated later. *Escherichia coli*: strain B, the American phage-test strain; 7/4 a mutant blocked above indole, supplied by Professor D. D. Woods; 366, a similar mutant of *E. coli* strain B, supplied by Professor K. C. Winkler.

*Media.* 'Defined' medium refers to a simple ammonia + glucose + salts medium containing m/10,000 cystine.

'Casein medium' contained buffer, 'vitamin-free' hydrolysed casein and glucose.

'Lactose-neutral red' medium was a nutrient agar based on the well-known MacConkey medium but without bile salts.

#### EXPERIMENTAL

##### *Extraction of tryptophan from Salmonella typhi*

Experiments were made to find whether tryptophan could be extracted from the bacteria merely by heating with water or neutral buffer at 100°. Gale (1947) found that other amino acids could thus be extracted from Gram-positive bacteria.

*Exp. 1. Extraction of tryptophan from Salmonella typhi by buffer at 100°.* One litre defined medium was inoculated with *S. typhi* Bact. 1 and agitated overnight at 37°. Next morning the opacity (Spekker) reading was 1·300, roughly equivalent to 2 mg. dry wt. bacteria/ml.; 880 ml. were centrifuged and the deposit made up in m/30 buffer (pH 7·2) to 10 ml. Thus the concentration of bacteria was now equivalent to c. 0·2 g. dry wt./ml. This suspension was then heated to 100° for 30 min., centrifuged and the supernatant fluid retained for estimation of the tryptophan content by the usual microbiological growth method. Seven different extracts were made as indicated in Table 1, differing in the concentration of bacteria in the extracts and in extraction time. Six were derived from *S. typhi* Bact. 1 and one from strain O 901 R. This latter organism was grown with tryptophan and was washed four times before extraction.

The organism used as growth-test object in the assay was *Salmonella typhi* O 901 R. This was grown overnight in defined medium + tryptophan. It was then washed 4 times in defined medium without tryptophan. The use of strain O 901 R as a test for tryptophan is, of course, open to criticism because it grows also in the presence of indole. It was, however, used as a convenience.

The criticism was met by showing that steam-distilled extracts did not differ significantly from undistilled and therefore did not contain indole. Further, one of the extracts was of O 901 R itself, which does not synthesize indole. In the assay, growth due to added extracts or tryptophan was estimated by opacity (Spekker) and converted to  $\mu\text{g.}$  tryptophan/ml. from a standard curve.

Table 1 shows that the extracts contained a material with the biological action of tryptophan. This was present both in bacteria without synthetic blocks and in O 901 R which is blocked below indole.

Table 1. *Estimation of tryptophan in extracts of Salmonella typhi Bact. 1 (1 to 6) and of S. typhi strain O 901 R (7)*

Extract no.	Concentration of extract	Extraction time (min.)	L-tryptophan in undiluted extract ( $\mu\text{g./ml.}$ )
1	10/1	5	$0.33 \pm 33 \%$
2	88/1	7	Doubtful
3	88/1	7	$0.58 \pm 24 \%$
4	88/1	7	Doubtful
5	88/1	30	$1.49 \pm 8 \%$
6	66/1	30	$1.26 \pm 9 \%$
7 (O 901 R)	88/1	30	$1.28 \pm 9 \%$

*Syntrophism of tryptophan-requiring Escherichia coli with Salmonella typhi*

The purpose of these experiments was to prove the presence of free tryptophan, not only in or on *Salmonella typhi*, but diffusing from it. The free tryptophan was to be detected by mixing with the culture a tryptophan-requiring coli mutant as indicator. These mutants have a synthetic block above indole and therefore accumulate indole in the culture. If syntrophic growth took place between the coli mutant and a typhoid strain under test in a medium free from indole or tryptophan, it should be an indication that the tryptophan required by the coli mutant was derived from the typhoid organisms.

The evidence of successful syntrophic growth was therefore as follows:

(1) The mixed culture in an indole- and tryptophan-free medium should accumulate indole and continue to do so on serial passage.

(2) The culture when plated on nutrient agar containing lactose and neutral red should grow differentiated colonies of coli and typhoid bacilli which could be isolated and tested to show that the bacteria were in the same metabolic condition as initially.

The following is an example of this type of experiment:

*Exp. 2. Syntrophism between Escherichia coli 7/4 and Salmonella typhi O 901 R.* *S. typhi* O 901 R and *E. coli* 7/4 were grown overnight in casein medium + m/10,000 indole or tryptophan, respectively. Next day, 1 drop of each culture was transferred separately to casein medium alone and for the syntrophic growth 1 drop of each together to casein medium. In 24 hr. the combined tube only had grown and one loop was taken to casein medium (1st transfer). Next day this had grown. The supernatant fluid contained indole



(Ehrlich's reagent) and both coli and typhoid organisms grew on the lactose + neutral red plates. These transfers were repeated 4 times and the last plating showed both typhoid and coli organisms. Colonies of the coli from the final neutral red plate were diluted and one loop taken to casein medium alone, to casein + indole and to casein + tryptophan. Growth appeared in all, showing that back-mutation had taken place. In the tube containing casein alone there was an accumulation of indole. This could only have been brought about by a coli back-mutant supplying the tryptophan.

This being so it might be argued that the growth of *Escherichia coli* 7/4 in syntrophism with the typhoid organism was due to tryptophan derived from a coli back-mutant and not from the typhoid bacilli. The experiment was therefore inconclusive so far as the typhoid organism was concerned.

The experiment was repeated using however *Salmonella typhi* Bact. 1 in place of *S. typhi* O901 R. The same procedure was followed with the same result to the 4th transfer. The coli and typhoid organisms were then isolated and each lightly inoculated into casein medium alone, casein + indole and casein + tryptophan. The coli grew only in tryptophan (indole +) while *S. typhi* Bact. 1, having no block, grew in all the media as usual. In this case therefore it seemed clear that tryptophan derived from the *S. typhi* Bact. 1 was the cause of the growth of *Escherichia coli* 7/4. This type of experiment is, however, apt to be abortive because of chance growth of coli back-mutants (cf. Winkler, van Doorn & Royers, 1952).

#### *Satellitism experiments*

In these experiments tryptophan produced by an organism was to be detected on an agar plate by production of a zone of growth of a tryptophan-requiring coli lawn surrounding an implanted patch of the organism under test. The *Escherichia coli* mutant 7/4 was used as the lawn and 9 different strains of *Salmonella typhi*: Vi strains, A, T, 1808; Smooth non-Vi, O901 S, P; Rough, O901 R, Bact. 1, ViIR. Bact. 1 had no synthetic block, but the others (except 1808) were blocked immediately below indole. Strain 1808 is discussed later.

All strains except 1808 produced zones of growth and therefore were judged to produce tryptophan. The procedure was as follows: *Escherichia coli* 7/4 was grown overnight in defined medium + tryptophan (M/10,000), washed twice in defined medium, diluted in the same to about  $10^7$  organisms/ml. and 0.5 ml. of this dilution flooded on to a casein plate. The fluid was then drained off and the plate dried on a hot-plate. On this lawn were dropped (c. 1/40 ml.) the typhoid cultures, each on a separate plate. They had been grown in casein medium with or without indole as necessary and washed once without indole. After drying the drops, the plates were incubated. Next day, the lawn remained free from obvious growth (or with a few back-mutants) but the plates had an odour of indole. The drops of suspension on the lawn had grown, those blocked using indole produced by the lawn. Controls dropped on a plate without lawn grew only in the case of *S. typhi* Bact. 1. In 2 days or perhaps later, a hazy zone appeared on the periphery of the patches which increased slowly to a well-marked halo. The controls without lawn showed nothing

similar. On examination a halo was found to consist of an increased surface growth and a subculture contained exclusively *E. coli* 7/4. Pl. 1, figs. 1 and 2, show the effect with *S. typhi* O901 R; figs. 3 and 4 with *S. typhi* Bact. 1.

These experiments were in agreement with a conclusion that *Salmonella typhi* produced excess tryptophan in the course of growth, even though the amount produced, as judged by the effect, was surprising in view of previous inability to obtain positive results. The minimal amount could be estimated roughly by measuring the diameters of the haloes and comparing with the rings produced by known quantities of tryptophan using the usual punched-out pit method. On this basis each drop of culture produced some 0.5–5.0  $\mu$ g. L-tryptophan. In fact, of course, with dilute tryptophan solutions in the pits the haloes were mere films, whereas with drops of culture there was a continuous production which resulted in a thick film without, however, increase in diameter. Thus the amount of tryptophan produced during growth was probably higher than this assessment suggested.

#### *The effect of indole*

*Salmonella typhi* strain 1808 was frequently isolated in 1946 in an epidemic in Wales. It has a nutritional disability which has not been fully analysed. In the absence of indole or tryptophan there is no growth, but with indole growth takes place slowly, though much more rapidly with tryptophan. In spite of the indole produced by lawns of *Escherichia coli* 7/4, *S. typhi* strain 1808 dropped on grew very little and produced no halo. It seemed that there was some difficulty in the synthesis of tryptophan and that this might be great enough to allow strain 1808 to be used as a lawn for indicating tryptophan-production. It had been noted that back-mutants of *E. coli* 7/4 had shown marked haloes on a lawn of *E. coli* 7/4 itself (Pl. 1, fig. 5) and it was concluded that *E. coli* B also must excrete tryptophan. *E. coli* B was therefore tried to test the value as a lawn of *S. typhi* 1808; but no haloes appeared in 6 days. From this it could be argued that a lawn of *S. typhi* 1808 was not stimulated by tryptophan from *E. coli* B, or that *E. coli* B did not excrete tryptophan. It was, however, recognized that the conditions of the two experiments were different. The back-mutant (equivalent to *E. coli* B) growing on a lawn of *E. coli* 7/4 and producing haloes was operating in an atmosphere containing indole, whereas *E. coli* B growing on a lawn of *S. typhi* 1808 and failing to produce a halo, was not. Exp. 3 was therefore carried out.

*Exp. 3. Effect of indole on tryptophan production by Escherichia coli B.* *Salmonella typhi* 1808, grown in casein medium + tryptophan, was washed once in casein medium, suspended in the same to a faint opacity and 0.5 ml. of this suspension flooded on each of four casein plates, which were then dried as usual. *E. coli* B was grown in casein medium, washed once and suspended in the same medium. Plates 1 and 2 carried the lawn only, plates 3 and 4 carried the lawn and drops of *E. coli* B suspension. Into the lids of plates 2 and 4 filter-papers were fitted carrying 12 drops of 0.01 M-indole. After 24 hr. of incubation there was no growth on plate 1; a slight film of growth on plate 2; drops without haloes on plate 3 and drops with well-marked haloes in a slight diffuse

growth on plate 4. Haloes appeared only when there was an atmosphere containing indole. Thus *E. coli* B exuded tryptophan which could be used by *S. typhi* 1808, but only in a detectable quantity in an atmosphere containing indole. Precisely the same result was obtained using *S. typhi* Bact. 1 as the implanted bacterium. In this case only 6 drops of indole were added to each filter-paper and the lawn was only very slightly grown. The haloes produced by *S. typhi* Bact. 1 in an atmosphere containing indole were well marked (Pl. 2, figs. 6, 7).

In both cases the increased production of tryptophan by growth in an atmosphere containing indole might well have been due to the use of this indole as a substrate of the indole  $\rightarrow$  tryptophan enzyme of the bacteria. Exp. 4 was carried out to test whether typhoid bacilli growing in excess indole did in fact produce more tryptophan.

*Exp. 4. The effect of indole on tryptophan synthesis by typhoid bacilli.* *Salmonella typhi* Bact. 1 was grown in casein medium + glucose in two 100 ml. lots, in 500 ml. flasks, one containing 0.001 M-indole, i.e. 10 times more than the concentration usually used for growth. The 500 ml. flasks were incubated stagnant overnight and the contents then centrifuged. Both supernatant fluids were steam-distilled until that containing added indole was free of it by colour test (Ehrlich). They were then autoclaved and assayed for tryptophan by a similar growth method to that used in Exp. 1, using, however, on this occasion *Escherichia coli* 7/4 as the test organism in defined medium.

The assay showed that the supernatant fluid from the culture grown without indole contained 0.4  $\mu\text{g}$ . L-tryptophan/ml.  $\pm 50\%$ , while that grown with indole contained 6.75  $\mu\text{g}$ ./ml.  $\pm 4\%$ . It was therefore concluded that the haloes observed on plates containing indole were due to a much larger tryptophan production under these conditions. Nevertheless, it was surprising that *Escherichia coli* B should produce tryptophan as a nutrient available to another organism when it contained an active tryptophanase. It was, however, recalled that Fildes (1938) had shown that indole inhibited tryptophanase.

The experiments of Fildes (1938) were repeated with current materials and more refined methods (indole estimations by Spekker and standard curves). *Escherichia coli* B grown in the presence of M/20,000 tryptophan was washed and suspended in buffer at a concentration *c.*  $4 \times 10^8$  organisms/ml., with L-tryptophan equivalent to 30  $\mu\text{g}$ . indole/ml. In 24 hr. 14  $\mu\text{g}$ . indole/ml. had been produced, i.e. about 50 % of the tryptophan had been degraded. At the same time the suspension was mixed with the same concentration of tryptophan + 1 equivalent of indole (30  $\mu\text{g}$ ./ml.). In 24 hr. there was no degradation of tryptophan. Thus the tryptophanase had been entirely inhibited by 1 equivalent of indole. The presence of less than 1 equivalent of indole inhibited roughly in proportion.

The action of indole in relation to tryptophan production by typhoid bacilli and *Escherichia coli* is therefore twofold. As a substrate of the synthetic enzyme it causes much larger quantities of tryptophan to be produced and as an inhibitor of tryptophanase it allows tryptophan produced by *E. coli* to survive. In the syntrophic growth experiments of Winkler *et al.* (1952) the



coli strain 366 produced excess indole, being blocked above indole. This excess indole caused over-production of tryptophan by coli 386 blocked below indole, and since the tryptophanase was inhibited also by the excess of indole, the tryptophan survived to act as nutrient to coli 366.

### CONCLUSIONS

It has been shown that strains of *Salmonella typhi* in which the indole  $\rightarrow$  tryptophan enzyme is unimpaired synthesize tryptophan in excess during growth. The amount produced may be so small as to be undetectable by the methods used, but when indole in excess is added to a culture, the production of tryptophan is much greater. Experiments with *Escherichia coli* also show an accumulation of tryptophan in cultures containing indole. The survival of the tryptophan in this case is due to inhibition of the tryptophanase enzyme by the indole.

I am indebted to Professor D. D. Woods, Oxford, and to Professor K. C. Winkler, Utrecht, for cultures; to Professor Winkler and Dr B. A. D. Stocker, London, for literature and to Dr D. Kay for help in various ways.

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### EXPLANATION OF PLATES

#### PLATE 1

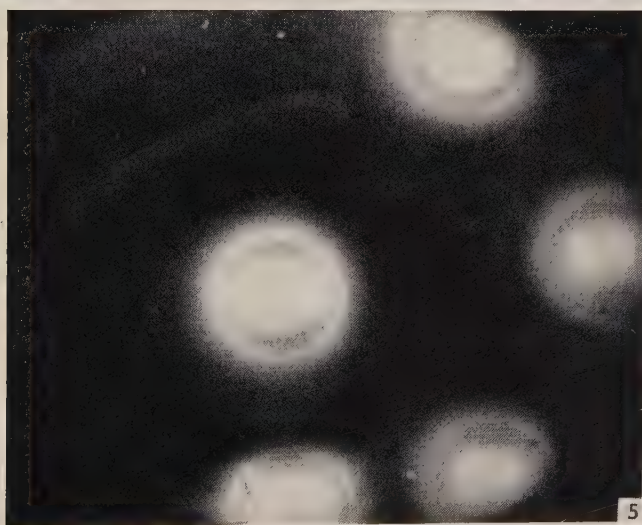
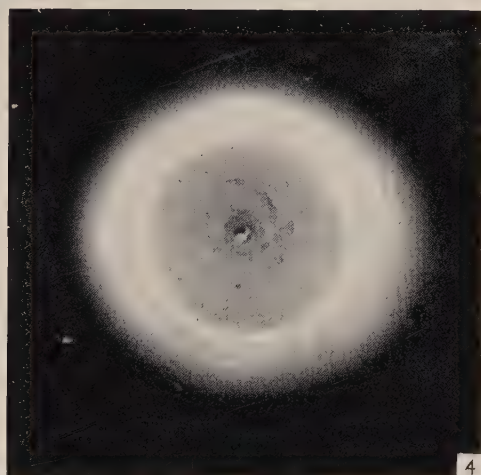
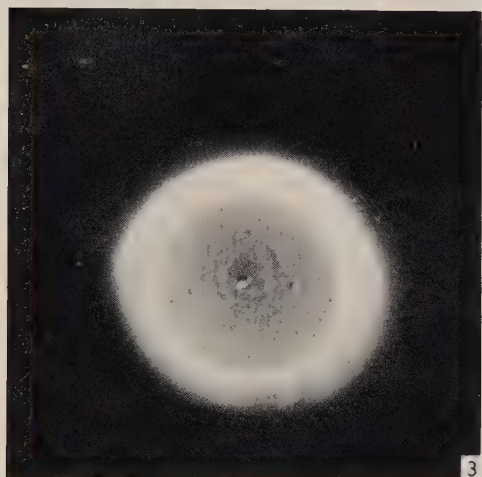
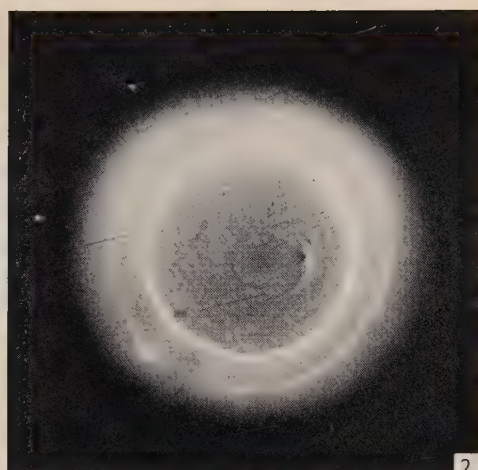
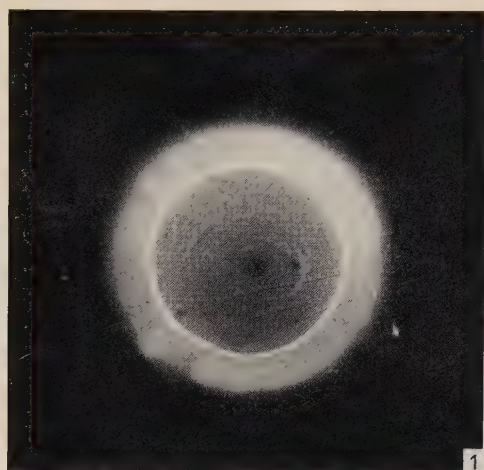
- Fig. 1. *Salmonella typhi* O901R growing on lawn of *Escherichia coli* 7/4; 2 days.  
 Fig. 2. Same plate at 3 days; same magnification.  
 Fig. 3. *Salmonella typhi*. Bact. 1 growing on lawn of *E. coli* 7/4, 2 days.  
 Fig. 4. Same plate at 3 days; same magnification.  
 Fig. 5. Back-mutants of *E. coli* 7/4 growing on lawn of *E. coli* 7/4; 6 days.

#### PLATE 2

- Fig. 6. *Salmonella typhi*. Bact. 1 growing on lawn of *S. typhi* 1808 in air; 22 hr.  
 Fig. 7. *S. typhi*. Bact. 1 growing on lawn of *S. typhi* 1808 in an atmosphere containing indole; 22 hr.

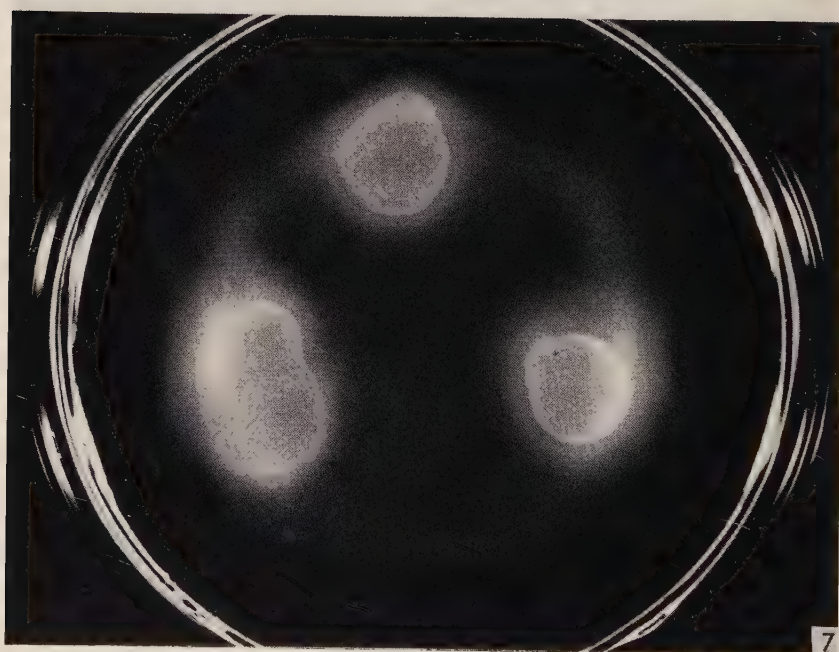
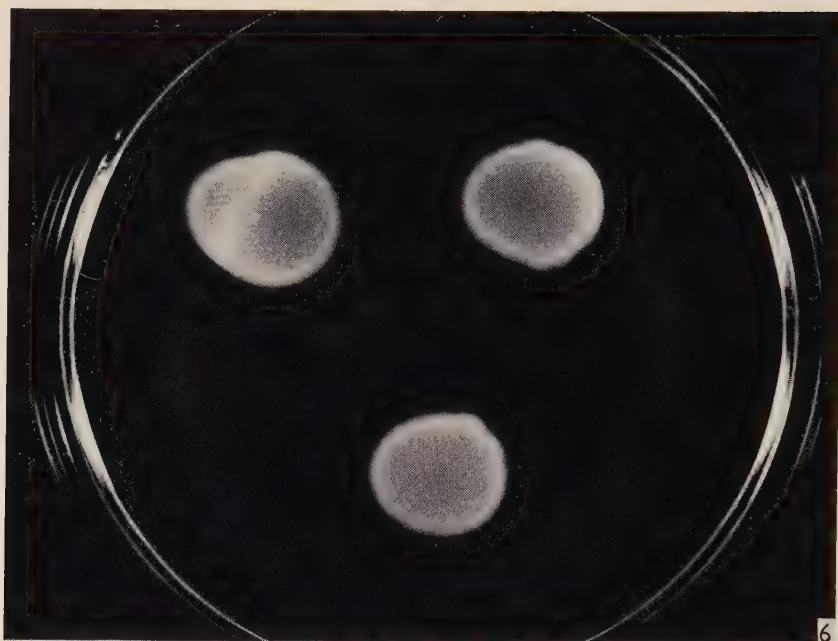
(Received 24 July 1956)





P. FIDES—ACCUMULATION OF TRYPTOPHAN IN CULTURES. PLATE I

(Facing p. 642)



P. FILDES—ACCUMULATION OF TRYPTOPHAN IN CULTURES. PLATE 2

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*Microbiologie.* By E. A. GRAY. Paris: Dunod. 1956. 248 pp.

*Microbiology and Pathology for Nurses.* By M. FROBISHER, L. SOMMERMEYER and R. H. GOODALE. 4th ed. London: W. B. Saunders Company Ltd. 1956. 845 pp. Price 45s. 6d.

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'Physiology and Voluntary Muscle'. *British Medical Bulletin.* London: The Medical Department of The British Council. 1956. 12, no. 3, 75 pp. Price 15s.





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## THE SOCIETY FOR GENERAL MICROBIOLOGY

*The Society for General Microbiology held its Twenty-second General Meeting in the Royal Institution, London, on Tuesday, Wednesday and Thursday, 10, 11 and 12 April 1956. The following communications were made:*

### COMMUNICATIONS

**An Environmentally Induced Transition from the Flagellated to the Non-flagellated State in *Salmonella*: the Fate of Parental Flagella at Cell Division.** BY C. QUADLING AND B. A. D. STOCKER (*The Lister Institute, London*)

When grown in digest broth at 37° *Salmonella typhimurium*, strain LT2, is peritrichously flagellated, with an average of about nine flagella per bacterium; but a culture grown at 44° is almost entirely non-flagellated. There is little change in the average number of flagella per bacterium when a 37°-grown culture is incubated at 44° for 4 hr., in the presence of a concentration of chloramphenicol which prevents synthesis of new flagella and arrests growth; it is inferred that flagella formed at 37° are not shed at 44°.

To determine the fate of parental flagella, the synthesis of new flagella was prevented by transferring a broth culture in logarithmic growth at 37° to 44°. The culture was maintained in logarithmic growth by periodic dilution with fresh broth; the mean generation time at both temperatures, inferred from turbidity measurements, was about 35 min. Samples were taken at intervals and fixed with formaldehyde; counts of numbers of flagella were made on 50–200 bacteria from each sample, in random fields of smears stained by Leifson's method. The average number of flagella *per flagellated bacterium* fell from an initial 9.1, to 4.8 after 60 min., and had reached 1.5 after 4 hr. growth at 44°; at this latter time about 70 % of the bacteria had no flagella, and most flagellated bacteria had but one flagellum each.

We infer that parental flagella are maintained during growth at 44°, and at each cell division are shared about equally between the two daughter cells. This suggests that growth of the cell wall is diffuse, and not entirely localized in a 'growing point'.

**The Properties of a Particular Type of Spontaneous Mutant of *Pasteurella pestis*.** BY S. JACKSON (*Microbiological Research Establishment, Porton, Wilts*)

When grown at 28° on a suitable synthetic agar medium containing haemin, many strains of *Pasteurella pestis* form dark brown colonies, due to the absorption of haemin. Prolonged incubation leads to the appearance of non-pigmented out-growths. From these, pure non-pigmented strains can be cultured.

Pigmented virulent strains give rise to non-pigmented mutants of reduced virulence. Thus, M3, a highly virulent pigmented strain, is invariably lethal to mice at intraperitoneal doses down to 100 organisms, whereas M7, a non-pigmented mutant of M3, only kills one to seven mice per batch of ten with doses ranging from  $10^2$  to  $10^6$  bacteria, there being no consistent increase in lethality with increase in dose over this wide range. M3 reaches an enormous population *in vivo*, about  $10^{10}$  bacteria being present per mouse at death. With M7, the population at death is very much smaller, and it has never been observed to exceed the order of  $10^8$  per mouse.

When non-toxic amounts of iron compounds are injected with the challenge doses of M7, its virulence pattern is transformed into that of M3, and the population at death reaches the enormous number characteristic of M3. The effect is specific for iron compounds.

Similar results have been obtained in experiments with non-pigmented mutants of other pigmented, virulent strains. All these strains can resist phagocytosis. Avirulent strains, whether pigmented or non-pigmented, remain avirulent in the presence of iron compounds; they cannot resist phagocytosis.

Apparently, for full virulence, *Pasteurella pestis* must, first, be able to resist phagocytosis, and, secondly, be able to produce pigmented colonies. This latter property is correlated with the ability to derive iron *in vivo*.

**Host Specificity of Myxoma Virus.** BY D. M. CHAPRONIERE AND C. H. ANDREWES (*National Institute for Medical Research, Mill Hill, London, N.W. 7*)

It has been found that some viruses are specific to one or a few closely related hosts, but will grow in cultures of tissues from normally resistant species; other anomalies also occur. Using methods of conventional tissue culture (to give unorganized, proliferating cells) and organ culture (to give organized cells with little or no proliferation) and the whole animal, we have tried to analyse host specificity with regard to myxoma virus.

Myxoma virus is very host specific, multiplying only in rabbits (*Sylvilagus* and *Oryctolagus*), very rarely in hares, and (in the laboratory) in brains of newborn mice and chick chorioallantoic membranes.

Tissue cultures of rabbit renal epithelium and heart fibroblasts supported virus growth and showed a characteristic cell change. In roller tube cultures of squirrel renal epithelium the virus also grew, causing cell necrosis and other changes, but adult squirrels showed only a transitory skin lesion when inoculated intradermally and no virus multiplication in skin or kidney after intradermal and intrarenal inoculation with virus passed 9–10 times in tissue cultures of squirrel kidney. From organ cultures of squirrel skin and kidney, virus has been recovered but there was no proven multiplication. Both human embryonic kidney epithelium and lung fibroblasts supported virus growth well but showed no apparent cellular changes. In organ cultures of human embryonic kidney the virus also grew well. The virus has also multiplied in

guinea-pig kidney cultures, but only from occasional experiments with rat kidney epithelium and chick chorioallantoic membrane tissue cultures was virus recovered.

The age of the animals and tissues used in these experiments was not uniform, some being embryonic, some neo-natal and others adult. This may have affected our results.

Thus, in tissues from different hosts in different conditions of organization, the myxoma virus shows varying behaviour.

**Genetic Transfer of Colicinogenic Properties by *Bact. coli*.** BY P. FREDERICQ  
(*University of Liège*)

Many strains of Enterobacteriaceae produce antibiotics named colicins. These colicins show very peculiar relations with bacteriophages. They are, however, inert chemical substances, proteins, devoid of the ability to reproduce. But, as colicinogenesis is an hereditary trait of the bacteria it was thought that the relations between colicins and phages may indicate relations between phages and the genetic factors governing colicin production. Transfer of these genetic factors was studied in transduction and recombination experiments.

(1) *Transduction*

Using a selective technique it was found that some colicinogenic strains may transduce their colicinogenic property to other non-colicinogenic strains. The transduced strains keep all their other original markers and differ only by the acquired hereditary property to produce colicin and by immunity to that particular colicin. This type of transduction is not effected by means of carried phages and seems to require conjugation of two cells as it succeeds only if the transducing strain is in the F+ state. It has not been possible to extract the transducing agent or to separate it from living cells.

(2) *Recombination*

Transduction of different colicinogenic properties to F+, F- and Hfr strains of *E. coli* K12 made possible the study of recombination. Transfer of the genetic determinants of colicin production is quite independent of all other known markers and is not affected by changing the selected markers. The colicinogenic property is always transmitted when carried by the F- parent and sometimes only when carried by the F+ parent. Colicin production decreases fertility in varying degrees according to the type of colicin produced. This decrease is more pronounced when colicinogenesis marks the F+ parent, in which case there may be complete sterility. This action upon fertility is attributed to induction during transfer and non-viability of the recombinants.



**Continuous Culture of Bacteria.** BY D. HERBERT (*Microbiological Research Department, Ministry of Supply, Porton, Wilts*)

The theoretical principles underlying the continuous culture of bacteria are discussed and the advantages of this technique, both as a research tool and for production purposes, are illustrated. Designs of continuous culture apparatus are discussed; they may be divided into the 'Chemostat' type (flow-rate controlled) and the 'Turbidostat' type (bacterial density controlled). Contrary to current opinion, these do not differ in principle; in each case the growth rate is equal to the dilution rate and a given bacterial density corresponds to a given steady-state nutrient concentration.

A laboratory continuous culture apparatus of the 'Chemostat' type is described. This consists of a culture vessel containing 500 ml. of culture, whose volume is maintained steady by a constant-level device. Sterile medium is pumped into the vessel at an accurately controlled rate by a 'peristaltic' pump (Sigmamotor Inc.) and culture is collected in a cooled receiver. Particular attention has been paid to obtaining adequate aeration of the culture; a specially designed stirrer and baffle system allows oxygen solution rates of 10 ml. O<sub>2</sub>/ml. culture/hr. to be obtained with an air-flow of 1 vol./vol. culture/min. This enables bacteria to be grown at high culture densities (> 15 mg./ml.) without aeration becoming growth-limiting. Under favourable conditions, outputs of over 250 g. dry wt. of bacteria/day can be produced with this small apparatus.

Air-flow through the apparatus is accurately stabilized, and provision is made for continuous recording of the O<sub>2</sub> and CO<sub>2</sub> contents of the outgoing air, using a paramagnetic O<sub>2</sub> analyser and an infra-red CO<sub>2</sub> analyser; continuous records of the O<sub>2</sub>-consumption and CO<sub>2</sub>-production of the growing bacteria are thus obtained. Results obtained with the apparatus are discussed.

**The Influence of Oxygen on the Metabolism of Growing Organisms Determined by means of the Continuous Culture Technique.** BY S. J. PIRT (*Microbiological Research Establishment, Ministry of Supply, Porton, Wilts*)

The carbon balances in growing cultures of *Aerobacter aerogenes* under aerobic conditions depend on the amount of available oxygen and the oxygen demand of the culture. The amount of available oxygen was determined by measuring the oxygen solution rate. The oxygen demand ( $-dc/dt$ ) of a growing culture in which growth is limited by the amount of carbon substrate is given by the relationship

$$-\frac{dc}{dt} = P\mu(s_0 - s),$$

where  $c$  is the concentration of dissolved oxygen,  $P$  is the number of moles of oxygen required per mole of substrate carbon utilized,  $\mu$  is the growth rate constant (i.e. growth rate per unit of cell mass),  $s_0$  is the initial concentration of substrate carbon,  $s$  is the concentration of carbon substrate at time  $t$ . The value of  $P$  is approximately 0.4 for fully aerobic conditions. *A. aerogenes* was



grown in continuous culture, the pH was 6.7 and glucose was the carbon source. Anaerobically cell synthesis and CO<sub>2</sub> production are at their minimum levels and most of the glucose carbon is converted into ethanol, formic acid, 2:3-butanediol, acetoin and acetic acid. A small supply of oxygen suppresses the formation of ethanol and formic acid but still permits the production of butanediol and acetoin and increases the proportion of glucose carbon converted to acetic acid, cells and CO<sub>2</sub>. A larger supply of oxygen suppresses the formation of butanediol and acetoin and still further increases the yields of cells and CO<sub>2</sub>. With an excess of oxygen available, provided the cell growth rate is not too near its maximum value, acetic acid production is suppressed and complete conversion of the glucose carbon into cells and CO<sub>2</sub> occurs. At cell growth rates very close to the maximum a part of the glucose is converted into acetic acid even with an excess of available oxygen.

**The Effect of Metabolite Analogues on some Virulence Factors of *Bacillus anthracis* in the Guinea-pig.** BY D. W. TEMPEST AND H. SMITH  
(Microbiological Research Establishment, Porton, Wilts)

To multiply *in vivo* and produce disease *Bacillus anthracis* must not only synthesize new cell material but also elaborate factors determining virulence, i.e. a protective capsule, and a toxin which initially acts as an aggressin and later kills the host. The metabolic processes of *B. anthracis in vivo* which underlie its virulence are being investigated.

During the last 9 hr. of anthrax there is a rapidly developing bacteraemia in which capsulated organisms are actively producing toxin. This afforded opportunity for studying the effect of metabolite analogues on processes concerned in growth and in the elaboration of capsule and toxin. Effects on the disease of side reactions between antimetabolite and host were minimized by direct intravenous injection of each compound and the short duration of each experiment.

8-Azaguanine was the most effective antimetabolite examined for inhibiting growth; hypoxanthine and adenine reversed this inhibition, whilst guanine and xanthine were ineffective. Ethionine, *p*-fluorophenylalanine and  $\alpha$ -amino-*n*-butyric acid were also bacteriostatic but not when administered with methionine, phenylalanine and alanine respectively. Toxin production was reduced when multiplication was inhibited but toxin synthesis per cell was not necessarily impaired.

Selective inhibition of toxin synthesis was observed when 2-thiouracil was injected; uracil, cytosine or thymine reversed this effect. Pyridine-3-sulphonic acid and 3-acetyl pyridine similarly restricted toxin synthesis without reducing growth rate or capsulation; in these cases nicotinamide restored toxin synthesis.

It has thus been possible not only to stop growth but also selectively to inhibit, and therefore obtain some insight into, the processes leading to toxin synthesis by *Bacillus anthracis*. Complete inhibition of capsule formation has not yet been achieved.

**Non-Flagellar Filamentous Appendages ('Fimbriae') and Haemagglutinating Activity in Dysentery Bacilli.** BY J. P. DUGUID AND R. R. GILLIES  
(Bacteriology Department, University of Edinburgh)

Electron-microscopic observations showed that 66 of 98 strains of *Shigella flexneri* possessed numerous fine filamentous appendages of the kind designated 'fimbriae' by Duguid, Smith, Dempster & Edmunds (1955). These strains underwent a ready, reversible mutation between a fimbriate form and a non-fimbriate form, the former becoming dominant after several successive subcultures in tubes of broth, and the latter after several subcultures on agar plates. Fimbriae were found in some strains of each of the serological types 1a, 2b, 3, 4a, 5, X and Y, obtained from the National Collection of Type Cultures, and in all of 47 freshly isolated strains of types 3, 4a and X.

The presence of fimbriae was invariably associated with the presence of haemagglutinating activity, suggesting that the fimbriae act as organs of attachment. Thus, all the fimbriate strains, when in their fimbriate phase, showed the power of adhering to and agglutinating the red blood cells of guinea-pig, fowl, horse, mouse, sheep, man and certain other animals. Moreover, they showed rapid adhesion to epithelial cells scraped from the mucous membrane of guinea-pig colon. Fimbriae, haemagglutinating activity and adhesiveness for intestinal epithelium were not observed in any strain of *Shigella sonnei*, *Sh. boydii* and *Sh. dysenteriae*. This suggests that the adhesive action of the fimbriae of *Sh. flexneri* is not of major importance in the pathogenesis of dysentery.

Pure antifimbrial serum was prepared by inoculating a rabbit with a live fimbriate culture and absorbing the resulting serum with a non-fimbriate culture of the same strain. Such a serum agglutinated fimbriate Flexner bacilli of all serological types equally, and also inhibited their haemagglutinating activity.

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**Lysis on Dilution of Bacteria Grown in High Salt Concentrations.** BY J. H. B. CHRISTIAN (Low Temperature Research Station, Cambridge)

Optical density measurements indicated that cells of the halophile *Vibrio costicolus* lysed readily when the osmotic pressure was suddenly reduced. Logarithmic phase cells from medium containing 1M-sodium chloride and suspended in 1M-sodium or lithium chloride began to lyse when the concentration fell below about 0.33M. On dilution from 1M-potassium or ammonium chloride, lysis began at concentrations above 0.8M, possibly because swelling rendered the plasma membrane permeable to these cations of smaller hydrated ionic radius. Older cells were more resistant to lysis.

The results were little affected by substitution of the chloride by other monovalent anions, by pH's from 4 to 10, or by prior incubation in  $10^{-5}$ M

mercuric chloride. Addition of cetyltrimethylammonium bromide (200  $\mu$ g./ml.) increased resistance to lysis in both sodium and potassium chloride solutions.

Lysis of cells from media containing from 0.5 to 3.0 M-sodium chloride commenced when the salt concentration fell to about one-third of that in which they were grown. This suggests that the osmotic pressure of the cell bears a constant relationship to that of the growth medium. Two non-halophilic species of *Vibrio* also lysed at salt concentrations of about half that in which they were grown, indicating that the same relationship between internal and external osmotic pressures may exist in both halophiles and non-halophiles.

Cells of *Vibrio costicollus* suspended in sodium chloride solutions of one-half the concentration in which they had been grown equilibrated rapidly with the new environment. This was shown by an increase in the resistance of the cells to subsequent osmotic shock.

**The Relationship between Arginine Breakdown and Motility in a Strain of *Pseudomonas*.** BY N. W. PRESTON, J. C. SHERRIS AND J. G. SHOESMITH  
(Department of Bacteriology, University of Manchester)

A strain of *Pseudomonas*, encountered as a contaminant, has been examined microscopically for motility in flat capillary tubes containing suspensions of the organism prepared from nutrient agar cultures. In aqueous suspension the bacteria became non-motile about 5 min. after introduction into the capillary tube except in a narrow zone near the air-suspension interface where active motility persisted for several days. When suspended in nutrient broth, the same initial events occurred; but, a few seconds after motility had ceased in the deeper parts of the suspension, it recommenced, and the organisms remained motile for about 40 min.

This restored 'deep' motility is apparently due to a different mechanism from the aerobic type. It could be activated by a solution of L-arginine in place of broth, and the duration of the restored motility was directly proportional to the arginine concentration in the suspending medium over a considerable range. Citrulline, ornithine, and eighteen other amino-acids were tested, but were unable to activate 'deep' motility. During the period of restored 'deep' motility, arginine was broken down at a steady rate, and was replaced by an equivalent concentration of ornithine. Motility ceased when all the arginine had disappeared. This breakdown of arginine did not begin until the initial period of aerobic motility had ended—a period which could be prolonged by deliberate aeration of the suspension.

It is not yet known whether the energy for 'deep' motility is provided by some stage in the conversion of arginine to ornithine, or whether this conversion limits the duration of motility by removing arginine from another system such as that found in invertebrate muscle.



**Biosynthetic Capabilities of the Parasitic Flagellate *Strigomonas (Herpetomonas) oncopelti*.** BY B. A. NEWTON (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The development of a simple synthetic medium which will support the growth of the parasitic flagellate *Strigomonas oncopelti* (Newton, 1956) permits for the first time a detailed study of the biosynthetic capabilities of an actively growing trypanosomid. The present communication describes the utilization of  $^{14}\text{C}$ -labelled acetate by *S. oncopelti* for the synthesis of cellular constituents.

Tracer amounts of acetate, labelled in the methyl or carboxyl position with  $^{14}\text{C}$ , were added to cultures of *Strigomonas oncopelti* growing in a synthetic medium containing glucose as principal energy source; after a further growth period corresponding to a doubling in cell count the cells were harvested, washed, fractionated and the distribution of  $^{14}\text{C}$  determined following the methods of McQuillen & Roberts (1954). The lipid fraction of the cells was found to contain 52 % of the total fixed  $^{14}\text{C}$  and the protein fraction 35 %, the remainder was distributed equally between the acid soluble cell constituents, an alcohol soluble protein fraction and the nucleic acid fraction of the cells. Further examination of the protein fraction after hydrolysis showed that both carbon atoms of acetate are utilized by *S. oncopelti* for the synthesis of glutamic and aspartic acids, alanine, threonine, tyrosine, lysine, arginine, valine, proline, phenylalanine, histidine and the leucines. The specific activities of glutamic acid, arginine and proline were found to be approximately equal, suggesting a relationship between these amino acids in that they derive the same number of carbon atoms from acetate; similarly, there appears to be a relationship between aspartic acid and threonine. Furthermore, it was found that glutamic acid, arginine and proline had considerably higher specific activities than aspartic acid, threonine and lysine, suggesting that acetate is used for synthesis in *S. oncopelti* by way of a tricarboxylic acid cycle rather than by a dicarboxylic acid cycle.

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MCQUILLEN, K. & ROBERTS, R. B. (1954). *J. biol. Chem.* **207**, 81.

**Sulphate-reducing Bacteria which are Deficient in Cytochrome.** BY J. R. POSTGATE (*Chemical Research Laboratory, Teddington, Middlesex*)

All mesophilic (30°) strains of *Desulphovibrio desulphuricans* so far examined contain cytochrome  $c_3$  and desulphoviridin (see Postgate, 1956). These pigments were not detected in six strains of the thermophile *D. thermodesulphuricans* grown at 50° and 55°. Traces of a protohaem in cell suspensions were indicated since a compound absorbing at 558  $\text{m}\mu$ . was formed with alkaline pyridine and dithionite. Two strains of *D. thermodesulphuricans* 'trained' to grow at 30° were also devoid of  $c_3$  and desulphoviridin; two strains of *D. desulphuricans* retained their pigments after 'training' to grow at 50°.



*Desulphovibrio desulphuricans* strain El Agheila Z required 10 mμmol. Fe/ml. for optimal growth in lactate-sulphate media; the cytochrome  $c_3$  content of the bacteria was proportional to the amount of Fe in the medium, but accounted for less than 1% of it on a molar basis. In pyruvate-sulphate media the strain had no absolute requirement for iron, but iron stimulated growth. This strain can grow in sulphate-deficient media if pyruvate is present; in such media it had no iron requirement and iron did not stimulate growth. The extents of growth were similar in iron-deficient pyruvate media whether sulphate were present or not.

Bacteria grown in iron-deficient pyruvate media of either kind contained no detectable cytochrome  $c_3$  and were unable to reduce sulphate in hydrogen; their contents of hydrogenase and desulphoviridin were reduced five- and twofold respectively. Hence cytochrome  $c_3$  deficiency is associated with inability to reduce sulphate in *Desulphovibrio desulphuricans* but not in *D. thermodesulphuricans*.

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**A Comparison of Cell-wall Composition in *Nocardia*, *Actinomyces*, *Mycobacterium* and *Propionibacterium*.** BY C. S. CUMMINS AND H. HARRIS (*Departments of Bacteriology and Biochemistry, The London Hospital Medical College*)

The cell-wall compositions of strains of *Nocardia*, *Actinomyces* and *Streptomyces* have been investigated by methods previously applied to other Gram-positive bacteria (Cummins & Harris, 1956). Among the 12 strains examined three groups could be clearly distinguished; these corresponded to micro-aerophilic, aerobic non-acid-fast, and aerobic acid-fast strains respectively.

The microaerophilic group comprised two strains of *Actinomyces israelii*, and the characteristic amino acids of the cell wall were alanine, glutamic acid and lysine. The other components in both these strains were hexosamine and galactose.

The aerobic non-acid fast group comprised three strains of *Actinomyces*, two of *Streptomyces* and one of *Nocardia gardneri*. The characteristic amino acids of the cell wall in these organisms were alanine, glutamic acid, glycine and LL-diamino-pimelic acid. Hexosamine was present, but no sugars, except in the cell-wall preparation from *N. gardneri*, which contained galactose.

In the aerobic acid-fast group, which consisted of four strains of *Nocardia*, the cell-wall fractions prepared in our usual manner showed a rather complex amino acid pattern involving many amino acids. However, when the material was extracted, before hydrolysis, with 0.5% KOH in alcohol, only three main amino acid components were found: alanine, glutamic acid and DAP (probably DL). The hydrolysates of these strains also contained large amounts of arabinose.

The pattern of cell-wall components found in the aerobic acid-fast group was thus similar to that which we had previously observed in *Corynebacterium*

*terium*. Preliminary investigations on three strains of *Mycobacterium* suggest that the same pattern obtains here also.

Four strains of *Propionibacterium* showed amino acid patterns closely resembling those found in the aerobic non-acid fast group of *Actinomycetes*.

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#### Some Observations on the Morphology and Taxonomy of *Lineola longa*.

By M. H. JEYNES (*Department of Bacteriology, University of Birmingham*)

The occurrence of changes in the characteristics of bacteria due to prolonged subculture is well known. A strain of *Lineola longa* which, on isolation in October 1953 possessed all the characters described by Pringsheim, was found, after weekly subculture for 12 months, to have lost its flagella, and obligate acetate requirements, and to have become Gram positive. The most surprising change was, however, the production of endospores on potato meal agar. Identical changes were also noted in the stock cultures maintained in the University of Cambridge.

Sporing bacilli, showing close resemblance to the changed *Lineola longa* cultures, were isolated from cow-dung and soil. Such organisms never occurred as contaminants.

Although the colony form and 'Stalactite' growth in fluid media were maintained and differed only slightly from freshly isolated strains of *Lineola longa*, the filaments possessed more frequent cross-walls and tended to be shorter.

The ejection of nuclear material by the turgid contents of the spore on treatment with 10% (v/v) nitric acid, a diagnostic test for *Bacillus* endospores, was found to occur in the *Lineola longa* spores.

The true relationship of *Lineola longa* to the genus *Bacillus* is thus clearly established. These filamentous organisms isolated from such habitats as cow-dung and soil, and from other similar situations, possessing typical colony form and giving 'Stalactite' growth in suitable liquid media, form a natural group within the genus.

*Lineola longa*, as originally described, is an extreme member of a continuous series of variants to be found in this group of *Bacillus* spp., which have probably arisen in response to environmental conditions.

#### The Submicroscopical Structure of Avian Tubercle Bacilli during Growth.

By E. M. BRIEGER AND AUDREY M. GLAUERT (*Papworth Hospital and Strangeways Research Laboratory, Cambridge*)

Two different types of reproduction occur in avian tubercle bacilli and there are corresponding differences in submicroscopical structure revealed by the ultra-thin sectioning technique.

In mycelial strains long filaments are formed which are composed of rows of individual cells. These cells are separated by cross-cell walls but often

remain attached to each other, and the outer cell wall is frequently continuous across the points of division. The cytoplasm of these cells varies in density but there are no well-defined light areas that can be identified with the 'nuclear apparatus' of the ordinary bacteria. During growth, buds originate in individual cells and the cytoplasm of the mother cell and bud are continuous in the early stages of bud formation. Dense, metaphosphate bodies are formed by the agglomeration of small, very dense granules and have no particular location.

In bacillary strains the short rods of the starting preparations have well-defined light areas containing dense threads and granules, similar to the nuclear apparatus of ordinary bacteria. After 24 hr. growth the rods elongate and there is a corresponding increase in size of the nuclear apparatus. The dense material within the light areas assumes a variety of configurations and is seen as threads, tubes and networks. Division by binary fission is observed and each daughter cell has a nuclear site. The cytoplasm appears as a uniformly granular matrix and contains larger, denser granules up to 300 Å. in diameter. At later stages of growth the bacilli continue to elongate and rows of spherical and ovoid bodies are formed in the cytoplasm. These bodies have limiting membranes, appear to lie outside the nuclear regions and have a uniform, granular cytoplasm. The nature of these bodies is being investigated.

**Some Submicroscopic Structural Features of Bacteria.** BY ERNA GROSS-BARD AND R. D. PRESTON (*Department of Botany, University of Leeds*)

Under the polarizing microscope, dry preparations of *Bacillus megaterium*, *B. subtilis*, *Escherichia coli* and *Proteus vulgaris* exhibited effects suggesting birefringence. As these were strongly reduced in preparations in liquids of varying refractive indices, these effects may have been caused mainly by depolarization, suggesting that the cell surface of these bacteria was smooth. True intrinsic birefringence, however, was observed in spores of *Bacillus alvei* and *B. cereus*, indicating orientation of molecular chains parallel to the spore surface. Thus, the polarizing microscope may prove useful for the elucidation of spore structure. It also greatly facilitates the observation of unstained preparations of vegetative cells.

X-ray diffraction studies on intact cells and cell walls of *Escherichia coli* demonstrated the presence of lipid rings with side spacings of 4.2 and 3.8 Å., mainly from the cell wall. The lipid crystallites are more than 250 Å. thick, thus thicker than the wall; the lipid chains cannot therefore lie parallel to the surface. Photographs of *Bacillus megaterium*, *B. subtilis* and *Proteus vulgaris* from intact cells pressed between glass plates and exposed with the X-ray beam parallel to the cell surfaces, showed meridional orientation of the lipid arcs. The lipid chains are thus at right angles to the cell surface as postulated by Hurst (1952) for *Escherichia coli*. Proteins, with spacings of around 4.5 Å., arranged at random, were identified in intact cells of the above bacteria. In cell walls of *Bacillus megaterium* and *B. subtilis* the protein rings were more intense and lipid rings less conspicuous than in *Escherichia*



*coli*. This fits in with the findings of Salton (1956) regarding a greater percentage of lipids in walls of Gram-negative than Gram-positive bacteria.

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**A Comparison of the Composition of and the Effect of Enzymes on the Spore Peptide and a Hexosamine Complex in Vegetative Cell Walls of *Bacillus subtilis*, *B. megatherium* and *B. cereus*. BY R. E. STRANGE AND F. A. DARK (*Microbiological Research Department, Porton, Wilts*)**

When the insoluble fraction from disintegrated, vegetative cells of *Bacillus cereus* (laboratory strain), was incubated with a filtered, aqueous extract of fresh, disintegrated resting spores of this organism, 50 % dried weight of the substrate was released as a soluble, hexosamine-containing complex (Powell & Strange, 1956). A smaller amount of similar material was released from the substrate suspended in water or buffer, but this was stopped by heating suspensions at 100° for 15 min. The lytic effect of the extract has now been observed using cell-wall preparations of vegetative *B. megatherium* and *B. subtilis* as substrate. Extract was freed from hexosamine by precipitation with an equal volume of McIlvaine's buffer, pH 3.0, or trichloroacetic acid (5 %, w/v) at 2°. The washed precipitate, dissolved in sodium bicarbonate solution, was dialysed at 2° without serious loss of activity. The optimum pH was 7.0–8.0 and activity was rapidly destroyed at 100°. A cell-wall preparation from vegetative *B. megatherium* was incubated in buffered, precipitated *B. cereus* spore extract with toluene at 37° for 48 hr. The soluble, non-dialysable material released contained  $\alpha$ -diaminopimelic acid, glutamic acid, alanine, glucosamine and an unidentified amino sugar. It contained more carbohydrate (excluding hexosamine) and had a higher mobility on paper electrophoresis at pH 8.6 than spore peptide. Similar results were obtained with cell-wall preparations of *B. cereus* and *B. subtilis*. This hexosamine complex differed from that liberated from cell walls by lysozyme or snail-gut enzymes in being non-dialysable and in giving a negative reaction for acetylhexosamine. Fragments released from it and spore peptide by enzymes from *Helix* included free acetylhexosamine.

It is possible that in resting spores the peptide is associated with the spore coat, the lytic system described being responsible for its liberation during disintegration or germination. The effect on vegetative cell walls suggests that it, or a similar system, has a role in lysing sporangia.

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**Distribution and Metabolism of the Stereoisomers of Diaminopimelic Acid in certain Bacteria.** BY D. S. HOARE AND ELIZABETH WORK  
(Department of Chemical Pathology, University College Hospital Medical School, London, W.C. 1)

Diaminopimelic acid (DAP) decarboxylase converts *meso*-DAP to L-lysine: DAP racemase interconverts the *meso* and LL isomers (Hoare & Work, 1955*a*). DD-DAP is metabolically inert. These enzymes and the DAP isomers were examined in acetone-dried cells from certain bacterial families selected because the commonly occurring *meso*-DAP was not invariably present (Hoare & Work, 1955*b*).

DAP was absent from most species of Micrococcaceae except for two types of staphylococcus (Cummins & Harris, unpubl.) which contained *meso*-DAP. High DAP decarboxylase and racemase activities were found in all species except *Sporasarcina ureae*, which lacked racemase.

Both DAP and the two enzymes were absent from the Streptococceae. Microbacteria and lactobacilli, which also lacked DAP (except for *Lactobacillus arabinosus* containing *meso*-DAP), had low levels of one or both enzymes. Propionibacteria (six species) contained LL-DAP and decarboxylase; racemase activities varied from zero to high.

Most Bacillaceae (eleven species) contained *meso*-DAP; *Clostridium welchii* had only the LL-isomer. All levels of racemase activities were found. Decarboxylase was absent from all species except *C. tetanii*.

The Actinomycetales were re-examined; their unknown amino acid 'A' (Work, 1953) has been identified as LL-DAP. Both mycobacteria and nocardia (seven species of each) contained high concentrations of *meso*-DAP, but a few nocardia had traces of LL-DAP. The few species examined for enzymes had moderate activities. One actinomycete contained a high level of LL-DAP and neither enzyme. Streptomyces (eight species) contained LL-DAP, but some also had smaller amounts of *meso* isomer. Micromonospora (three species) had both *meso* and LL isomers and also traces of DD-DAP (identified by stability to enzymes).

The distribution of DAP and its isomers in whole cells, which is similar to that in cell walls (Cummins & Harris, 1956*a, b*), cannot be correlated with activities of either DAP racemase or DAP carboxylase.

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**Morphological Observations on Vegetative Nuclei of *Phycomyces*, *Mucor*, *Saprolegnia* and other Fungi.** BY C. F. ROBINOW (University of Western Ontario, Canada)

**Electron Microscopy of L-forms Induced in *Vibrio* spp. by the Action of Penicillia.** BY PHYLLIS PEASE (*University of Birmingham*)

A FILM

**Genesis of Bacterial Flagella.** BY A. PIJPER (*Institute for Pathology, Pretoria*)

The flagella of a *Spirillum*, clear-cut, horny-looking and with a set curve, appear first and contrast with the fuzzy-looking straight tail dragged along by other motile bacteria (*Salmonellae*, *Proteus*, *Caryophanon*). These other motile bacteria are shown wriggling their flexuous bodies, sometimes with and sometimes without the straight tails. At high magnification the wriggling looks a cell-wall function. In time the straight tails are seen to stiffen into helices, maintaining however their over-all length. These helices are commonly called flagella. A given bacterial strain often shows helices of two wavelengths, one always twice the other ('biplicity'). This stiffening does not interfere with motility. Motility stops when in due course the bodies also become rigid. Splitting of the helices is seen to introduce various types of flagellation. Tensions apparently created in the helices through the transition from straight tail to helix (a very violent happening in short-wave helices) cause the helices to whirl for a time. This causes maelstroms around anchored rigid bodies and pushes around free rigid bodies, simulating motility. True motility is again shown as a wriggling of the body. Conclusion: helical bacterial flagella are stiffened straight tails and true motility being a cell-wall function knows them not.

*Followed by a Symposium on*

**BACTERIAL ANATOMY**

**Cellular Organization in Bacteria.** BY K. A. BISSET (*University of Birmingham*)

**Bacterial Flagella: Morphology, Constitution and Inheritance.** BY B. A. D. STOCKER (*Lister Institute, London*)

**Bacterial Capsules and their Relation to the Cell Wall.** BY J. TOMCSIK (*Institute of Hygiene and Bacteriology, University of Basel*)

**Capsule Formation and Glutamyl Polypeptide Synthesis by *Bacillus anthracis* and *Bacillus subtilis*.** BY C. B. THORNE (*Camp Detrick, Maryland, U.S.A.*)

**Bacterial Cell Walls.** BY M. R. J. SALTON (*University of Cambridge*)

**Bacterial Protoplasts; their Formation and Characteristics.** BY C. WEIBULL (*Biochemical Institute, Uppsala*)

**Capabilities of Bacterial Protoplasts.** BY K. MCQUILLEN (*University of Cambridge*)

**Osmotic Function and Structures in Bacteria.** BY P. D. MITCHELL and JENNIFER MOYLE (*University of Cambridge*)

- The Chromatin Bodies of Bacteria.** By C. F. ROBINOW (*University of Western Ontario, Canada*)
- Bacterial Chromosomes and their Mechanism of Division.** By E. D. DELAMATER (*University of Pennsylvania, U.S.A.*)
- On the Organization of the 'Nuclear Material' in *Salmonella typhimurium*.**  
By O. MAALØE and A. BIRCH-ANDERSEN (*Statens Seruminstitut, Copenhagen*)
- Chromosomes in Micro-organisms.** By C. G. ELLIOTT (*University of Glasgow*)
- Organization of Bacterial Cytoplasm.** By J. R. G. BRADFIELD (*Cavendish Laboratory, University of Cambridge*)
- Inclusions in Bacteria.** By C. L. HANNAY (*Science Service Laboratory, Canadian Department of Agriculture*)
- The Structure and Development of the Induced Long Forms of Bacteria.**  
By W. H. HUGHES (*Wright-Fleming Institute of Microbiology, London*)

#### ERRATA

Proceedings of 21st Meeting, *J. gen. Microbiol.* vol. 14 (1956)

Page iii. Communication by E. J. L. Lowbury and L. Hurst  
for '*Streptomyces*' read '*Streptococcus*' throughout

Page viii. Communication by J. WOLF and S. A. Z. MAHMOUD  
l. 5 of abstract: for '0.1 g.' read '0.1 mg.'





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